

University of Alberta

Combined Immune Gene Therapy for Malignant Melanoma

by

Dr. Edward Bernardo Solano ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial

fulfillment of the requirements for the degree of *Master of Science*

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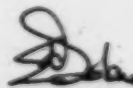
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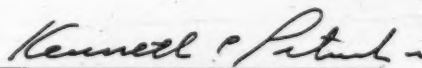
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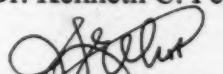
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Table of Contents

Chapter 1.	General Introduction	Page
I.	Introduction to Melanoma	2-10
II.	Gene Therapy	10-19
III.	Immunomodulating Molecules	19-30
IV.	Bibliography	31-45
Chapter 2.	Retroviral-Based Combined Immune Gene Therapy for Human Malignant Melanoma in a Hu-PBL-SCID/bg Mouse Model	
I.	Introduction	47-49
II.	Methods	49-55
III.	Results	55-59
IV.	Discussion	59-63
V.	Conclusions	63-64
VI.	Bibliography	77-81
Chapter 3.	Retroviral-Mediated Transduction of Human Melanoma Cells with CD154	
I.	Introduction	83-85
II.	Methods	85-90
III.	Results	90-94
IV.	Discussion	94-98
V.	Conclusions	98
VI.	Bibliography	115-119
Chapter 4.	General Discussion and Conclusions	
I.	General Comments	121-124
II.	Future Directions	124-125
III.	Conclusions	126
IV.	Bibliography	127-128

Table of Contents

	Page
Appendix 1 The AJCC Staging System for Melanoma	129-130
Appendix 2 Cloning CD154	131-134
Appendix 3 Generation of pCSD7-CD154	135

List of Tables

		Page
Table 2-1	Immunohistochemical analysis of cultured melanoma cells.	65
Table 2-2	Expression of common cell surface molecules on cultured melanoma cells.	65
Table 2-3	Expression of CD86 following transduction with pLSN-b70 or pLSN-BG9.	66
Table 2-4	Expression of GM-CSF following transduction with pLSN-GM1 or pLSN-BG9.	67
Table 2-5	Expression of IL-12 following transduction with pLSN-IL-12.	67
Table 3-1	Expression of CD40 and CD154 on WT and CD154-transduced melanoma cells.	99
Table 3-2	Lymphocyte marker expression on PBMCs stimulated with WT or CD154-transduced melanoma cells.	99
Table 3-3	Expression of HLA and B7 molecules on DC following stimulation with WT melanoma cells or CD154-transductants.	100

List of Figures

		Page
Figure 2-1	Diagrams of retroviral vectors	68
Figure 2-2	Flow cytometry histograms assaying B7-2 expression following transduction with pLSN-BG9 or pLSN-B70.	69
Figure 2-3	Stimulation of peripheral blood mononuclear cells with wild-type and transduced forms of Ed141.Mel.	70
Figure 2-4	Cytotoxic potential of Ed141.Mel-stimulated PBMCs.	71
Figure 2-5	Ed141A1.Mel vaccination experiment.	72
Figure 2-6	Ed141A1.Mel 2 nd allogeneic vaccination experiment.	73
Figure 2-7	Ed162.Mel allogeneic vaccination experiment.	74
Figure 2-8	Ed176.Mel allogeneic vaccination experiment.	75
Figure 2-9	Ed176.Mel autologous vaccination experiment.	76
Figure 3-1	Gel electrophoresis of the RT-PCR gene product.	101
Figure 3-2	Diagrammatic representation of pCSD7.	102
Figure 3-3	Diagrammatic representation of pLSN-BG9.	103
Figure 3-4	The sequence of cloned CD154.	104
Figure 3-5	CD154 immunohistochemical staining patterns of WT and transduced Ed141.Mel cells.	105

Figure 3-6	CD154 immunohistochemical staining patterns of WT and transduced SKMel28 cells.	106
Figure 3-7	CD154 immunohistochemical staining patterns of WT and transduced Ed176.Mel cells.	107
Figure 3-8	CD154 immunohistochemical staining patterns of WT and transduced Ed222.Mel cells.	108
Figure 3-9	CD154 immunohistochemical staining patterns of WT and transduced Ed224.Mel cells.	109
Figure 3-10	Proliferative response of PBMCs following co-culture with WT melanoma cells and CD154-transductants.	110
Figure 3-11	Enhancement of the proliferative response in the presence of CD154.	111
Figure 3-12	Flow cytometry histograms of DC cell expression of Class I HLA molecules following co-culture with WT melanoma cells and CD154-transductants.	112
Figure 3-13	Flow cytometry histograms of DC cell expression of Class II HLA molecules following co-culture with WT melanoma cells and CD154-transductants.	113
Figure 3-12	Flow cytometry histograms of DC cell expression of B7-2 molecules following co-culture with WT melanoma cells and CD154-transductants.	114

List of Abbreviations

APC	Antigen presenting cell.
AJCC	American Joint Committee on Cancer
BCG	bacille Calmette-Guerin
CD	Cluster of differentiation (antigens)
CMI	Cell-mediated immunity
CTL	Cytotoxic T-Lymphocyte
CTLA-4	Cytotoxic T-lymphocyte associated Molecule
DC	Dendritic cell.
FITC	Fluorescein IsoThioCyanate
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GMTC	Genetically modified tumour cell.
GMTV	Genetically modified tumour vaccine
HBSS	Hank's Balanced Saline Solution
HLA	Human Leukocyte Antigen
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IL	Interleukin
LTR	Long Terminal Repeats
MAGE	Melanoma Associated Genes
MHC	Major Histocompatibility (Antigen)
NK	Natural Killer (cell)
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RPE	Rhodamine
SCID	Severe Combined Immunodeficiency
TNF	Tumour Necrosis Factor
TIL	Tumour Infiltrating Leukocytes
TGF	Transforming Growth Factor
WT	Wild-type.

Abstract

Malignant melanoma is a neoplasm of increasing significance. This thesis explores a novel therapeutic strategy – to transfer into these cells genes encoding pro-inflammatory molecules and then using these genetically modified tumour cells as vaccines to potentiate a host's immune response to *in situ* melanoma.

Human melanoma cells were manipulated by insertion of genes encoding for Granulocyte Macrophage-Colony Stimulating Factor, Interleukin-12, and the T-cell co-stimulatory molecule B7-2 through the use of previously engineered retroviral vectors. *In vitro* experiments demonstrated up-regulation of cell-mediated immunity following stimulation with modified tumour cells. In subsequent attempts to inhibit the growth of wild-type human melanoma in human lymphocyte immune reconstituted severe combined immunodeficient mice through therapeutic vaccination, no significant treatment effects could be demonstrated.

In a separate set of experiments, the gene for CD154 was cloned and genetically engineered into a retroviral vector, which was subsequently used to transfer the CD154 gene into cultured melanoma cells. These modified cells demonstrated the capability to induce up-regulation of T-cell proliferation and dendritic cell activation.

Based on these studies, further evidence is provided that immune responses can be generated against human melanoma. Thus, immune gene therapy for human melanoma is both feasible and promising.

Chapter 1

General Introduction

I. Introduction

A. Definition, Epidemiology, and Etiology

Malignant Melanoma is a malignancy of melanocytes. These are cells that make the pigment melanin and are of neural crest origin. Melanocytes are found primarily in the skin, thus, this malignancy occurs predominantly in the skin (primary cutaneous melanoma). However, melanocytes are also found in mucous membranes, the upper esophagus, meninges, perianal and genital regions, and the eye; thus, melanoma can occur at all of these other sites as well. ([1] [2]).

In North America, melanoma is the 7th leading cause of cancer with an estimated lifetime risk of about 1 %. The incidence is rapidly rising both in North America and worldwide, particularly in Caucasian populations, with the highest incidence in Queensland, Australia where the annual incidence is almost 40/100,000. Asian and Black populations have much lower incidence rates. According to SEER (US Surveillance, Epidemiology, and End-Results program) data, mortality has increased by 35.2% between 1973 and 1991; however, the overall 5 year survival rates have also been increasing (in 1974 the 5 year survival rate was 79.7%, while in 1991, this had risen to 85.1%). This phenomenon is likely related to better screening leading to earlier diagnosis, "lead time bias," and improvements in treatment. ([3] [4])

Many risk factors have been identified ([5] [6] [3] [1]). Of these, sunlight exposure is the most important because it is the only one that is potentially preventable. Data implicate both UVA and UVB. The exact relationship remains to be fully elucidated, but it is known that UV radiation inflicts DNA damage, which may lead to somatic mutations. Accumulation of such mutations can eventually lead to

carcinogenesis. Unlike the other major skin cancers (squamous cell and basal cell carcinomas), melanoma risk correlates more with intense, intermittent exposure rather than chronic exposure. In particular, numerous sunburns in childhood imparts significant risk. Other associations include pigment traits (blue/green eyes, fair/red hair, pale complexion, freckling) and a family history (5-10% of cases). Precursor lesions (benign melanocytic nevi, giant congenital nevi, spitz nevi, and the atypical mole syndrome) may degenerate into melanomas; however, it is felt that most of these lesions are simply markers for the disease and that only 30% of melanomas arise from nevi.

B. Natural History and Staging

The majority of lesions are detected at an early stage and are promptly dealt with by surgical excision. If allowed to progress, metastases via hematogenous and lymphatic routes can involve almost any organ in the body. The most commonly involved sites are distant subcutaneous sites, lymph nodes, the gastrointestinal tract, liver, lungs, and the central nervous system. Autopsies performed on patients dying of metastatic melanoma often show near complete replacement of many visceral organs.

The most current staging system was developed by collaboration between the American Joint Committee on Cancer (AJCC) and the Union Internationale Contre le Cancer (UICC) (Appendix 3). Briefly, Stage I represents a primary melanoma between 0.76-1.5 mm thick and/or Clark's level II-III invasion with no nodal or systemic metastases. Stage II represents a primary melanoma between 1.51-4.0 mm thick and/or Clark's level IV-V invasion or satellite lesions within 2cm of the primary tumor, but without nodal or systemic metastases. Stage III represents regional lymph node

metastases and/or in-transit metastases. Stage IV represents the presence of systemic metastases.

C. Conventional Therapy

Surgical excision remains the most effective form of therapy for malignant melanoma, but only if surgery is performed at an early stage. 5-year survival for Stage I melanoma is excellent following appropriate surgical resection (>90%); however, Stage IV melanoma has a dismal prognosis regardless of the treatment provided (~5% 5-year survival, 6-11 month median survival). ([2] [5] [6] [7])

As with other malignancies, once the disease spreads beyond the original local confines, systemic therapy is necessary. Melanoma is a notoriously radioresistant tumor, thus, it has no role as adjuvant therapy ([1] [2]). The mainstay of adjuvant therapy has traditionally been chemotherapy. Unfortunately, the results of adjuvant chemotherapy have been overwhelmingly disappointing. The best single agent appears to be Dacarbazine (DTIC), with response rates of 15-25%. Moreover, this response is usually short-lived (5-6 months) and only rarely is the response complete (5%). Overall, only 1-2% experience long-term complete responses. Other agents have been tested including nitrosoureas, vinca alkaloids, Taxol, temozolimide, and others; however, the results with these agents are even more disappointing. Combination regimens have led to higher response rates (up to 40%); however, randomized control trials have yet to show significant survival benefits. ([5])

Because melanoma appears to be an inherently immunogenic tumour, immune-based therapies have been intensely studied over the past three decades, beginning with

BCG usage in the 1960s. BCG (bacille Calmette Guerin) is a non-specific activator of the immune system. Morton et al ([8]) demonstrated that up to 90% of lesions regressed following direct intralesional injection of BCG. In fact, some non-injected lesions also regressed. Patients treated in this manner developed antibodies to melanoma antigens and areas of regression were associated with intense lymphocytic infiltration. Unfortunately, subsequent studies failed to show any survival benefit from this relatively crude form of therapy ([9] [10]). Other investigators have also utilized BCG as complementary adjuvant therapy given along with anti-tumour vaccines ([11]).

Recent studies suggest that IFN- α may have a major therapeutic role ([12]). This cytokine is known to enhance cell-mediated responses, enhance HLA expression, decrease oncogene expression in some tumors, and possess anti-proliferative properties (arrests the cell cycle at G₀ and decreases protein synthesis). The Eastern Cooperative Oncology Group (ECOG) recently concluded a multicentre randomized control trial (EST 1684) which examined the use of high-dose IFN- α 2b in Stage IIb and Stage III melanoma patients following surgery. Significant increases were seen in median disease free survival (from 1 to 1.7 years) and in overall survival (from 2.5 to 3.8 years). To date this is the only form of adjuvant therapy for which level 1 evidence exists showing a survival benefit, albeit only a modest increase in survival. A limiting treatment factor is the toxicity associated with IFN- α , which includes constitutional symptoms (fever, chills, fatigue, malaise), neuropsychiatric symptoms (depression), myelosuppression, and hepatotoxicity. In fact, in the ECOG study, 67% of patients had severe toxicity, 9% had life-threatening toxicity, and 2 out of 143 patients died of hepatotoxicity directly attributable to IFN- α therapy. Another treatment-related factor is cost, which is

estimated to be \$30,000 per year ([13]). Other regimens have been tested including lower-dose IFN- α with and without IL-2 ([14] [15]); however, these regimens did not have as dramatic an effect as did high-dose IFN- α 2b. The ECOG protocol has received FDA approval for use as adjuvant therapy for Stage IIb and Stage III melanoma. Beneficial adjuvant therapy for Stage IV melanoma has yet to be developed.

D. Melanoma Immunology

Much has been learned over the past 10-15 years with regards to the immunobiology of melanoma. Early observations of the clinical course of persons afflicted with melanoma suggested that the immune system plays an important role in this disease. For instance, numerous cases of spontaneous regression have been documented ([16]), and, in lesions showing areas of regression, histologic examination demonstrated an intense lymphocytic infiltration ([8]). Secondly, in approximately 5% of cases, patients will present with a metastasis, but the primary remains occult. It is postulated that in at least some of these cases, spontaneous regression has occurred ([17]). Thirdly, there have been suggestions that the autoimmune destruction of melanocytes in the disease state of vitiligo can be explained by normal immune surveillance for melanoma. A study by Koh et al suggested that patients who had both melanoma and vitiligo leukoderma had a better prognosis than melanoma patients without vitiligo([18]).

Central to the theories supporting immunotherapy is the notion that tumor cells, being altered self cells, express antigens which can be recognized and acted upon by effectors of the immune system ([19]). Several studies have confirmed the immunogenicity of melanoma cells. For instance, antibodies reactive to autologous

melanoma cells have been identified in the peripheral circulation of melanoma patients ([20]). Furthermore, cytotoxic T-lymphocytes (CTLs) reactive to melanoma cells have been isolated in the peripheral circulation and within tumours themselves ([21] [22, 23] [24] [25]). These lymphocytes are referred to as tumor infiltrating lymphocytes (TILs) and have been shown to be reactive against melanoma antigen epitopes. They can be expanded *in vitro* in the presence of interleukin-2 (IL-2) and used in adoptive therapy strategies ([24]).

Tumour-specific targets have yet to be discovered for any tumour. All of the known tumour markers are referred to as tumour-associated i.e. they may be found in non-malignant as well as malignant tissue. A number of melanoma associated antigens have been described ([26] [27] [28] [29]). Early experiments suggested the presence of at least 3 antigens presented in association with HLA-A2 (a Class I MHC molecule) on SKMel29 cells ([28]). Autologous T cell clones recognized these antigens in an MHC dependent manner in that CTL activity could be blocked by monoclonal antibodies to HLA-A2.

The first gene to be identified as coding for a melanoma associated antigen was the MAGE-1 gene, which codes for an antigen originally labeled MZ2-E ([29]). MAGE is a gene family (MAGE-1,-2,-3), all of which code for melanoma antigens. These genes are not usually expressed in normal tissue other than the testis. However, other tumors, such as lung, brain, and breast cancer, can variably express these antigens ([26]).

Another set of melanoma-associated antigens have been categorized as melanoma differentiation antigens, which are expressed on the majority, but not all melanomas. Amongst normal cells, these are expressed only on melanocytes. The first of these to be

identified was the tyrosinase gene product ([26]). The other melanoma differentiation antigens include gp 75 ([27]), gp 100 ([30]), and Melan A/MART-1 ([31] [32]). CTL activity against all of these antigens has been demonstrated. Additionally, an experiment involving cloning the Melan A/MART-1 gene into melanoma cells rendered these cells more immunogenic ([31]).

A third group of melanoma-associated antigens are those that arise from point mutations. These are not as well characterized as the others, but they also demonstrate immunogenicity ([33]).

Because melanoma cells may share antigens with normal cells, the potential for autoimmunity exists. It has been proposed that natural effector cells against melanoma antigens are indeed present, but that they have very weak activity and the immune response obtunded ([34]).

E. Immune Evasion Mechanisms

Non-immunogenic tumors express tumor antigens, but they exert various mechanisms to prevent the generation of an effective immune response ([35]). One such mechanism is down-regulation of MHC Class I molecules ([36] [37]). These molecules are expressed on most nucleated cells, including most epithelial-based tumor cells. Their importance is underscored by the concept of MHC restriction, whereby T cells recognize their nominal antigen, but only if that antigen is presented in the context of an appropriate MHC molecule. Specifically, CD8+ cytotoxic T lymphocytes can recognize their nominal antigen only if this antigen is presented in conjunction with a Class I MHC molecule ([34]). Loss of MHC/HLA Class I expression has been described

in melanoma cells and has been shown to be due to a defective β_2 microglobulin gene ([38] [39]) (the Class I α chain normally forms a complex with β_2 microglobulin on the cell surface). Down-regulation of MHC Class I molecules is seen in approximately 20-40% of melanomas ([40]). The immunogenicity of tumors lacking Class I molecules can be increased following transfection of these same cells with Class I MHC genes ([36] [37]) or through transfection with the IFN- γ gene which secondarily upregulates expression of Class I and Class II HLA molecules ([20]).

Additionally, with melanoma progression, tumor cells may express lower levels of common melanoma-associated antigens. Jager et al demonstrated that serial biopsies from patients with clinically progressive melanoma showed gradual loss of expression of Melan A/MART-1 and tyrosinase ([41]), rendering them less immunogenic.

Another means of tempering the immune response is through the production of immunoinhibitory cytokines by tumour cells. In the case of melanoma, IL-10 appears to be important. This cytokine is elaborated by melanoma cells and the IL-10 receptor has been found on the surface of melanoma cells. IL-10 down-regulates expression of HLA Class I, Class II, and ICAM on human melanoma cells and acts as an autocrine growth factor ([42]). TGF- β 2 may have a similar role. It has been suggested that TGF- β 2 may be over-expressed by some patients with disseminated malignant melanoma ([43]). This molecule is known to down-regulate local immune responses in certain malignancies, such as glioblastoma ([44]).

One of the proposed mechanisms of anti-tumour immunity is induction of apoptosis in the tumour cell. The prototypical system is the FAS:FAS-Ligand interaction. T-cells expressing FAS-Ligand are thought to induce apoptosis through

interaction with FAS on the tumour cell surface. However, some investigators have demonstrated that though some melanomas express the FAS molecule, they may not necessarily be susceptible to FAS-Ligand ([45]). Some melanoma strains also express FAS-Ligand and it is proposed that such FAS-Ligand bearing tumour cells could actively delete FAS-bearing lymphocytes within its vicinity thus protecting itself from CTL-mediated killing ([46], [47]). A separate apoptosis pathway may be more likely. TRAIL (TNF-related apoptosis-inducing ligand) has been shown to be effective at inducing apoptosis ([48]). However, even in this scenario, immune-evasion mechanisms have been proposed including tumour expression of intracellular apoptosis inhibitors such as FLIP (FLICE-inhibitory protein) and/or decoy/anti-apoptotic receptors (TRAIL-R3 and TRAIL-R4) ([49]).

II. Gene Therapy

A. General

In the broadest sense, gene therapy is defined as manipulation of genetic material for the purpose of treating disease. Immunogene therapy refers to the manipulation of those genes whose products directly or indirectly affect immunity. The first reported cure of a genetic disease involved reversal of dwarfism in mice due to growth hormone deficiency ([50]). The gene for growth hormone was inserted into germ line cells, thus overcoming dwarfism. Unfortunately, gigantism resulted due to the uncontrolled overexpression of growth hormone. The first use of somatic gene therapy for human disease occurred in 1990 ([51]). The disease treated was a form of severe combined immunodeficiency known as adenosine deaminase (ADA) deficiency. ADA is crucial to

T and B cell development; thus, in a deficient state, functional T and B cells do not develop. The ADA gene was retrovirally transduced into the lymphocytes of two patients with ADA deficiency. These lymphocytes were re-infused back into the patients resulting in improved immune function. ADA deficiency is an example of a disease caused by a deficiency or defect of a single gene. Other such diseases include Lesch-Nyhan syndrome, LDL receptor deficiency, and α -1 antitrypsin deficiency. These were natural early targets for gene therapy. ([52])

In cancer gene therapy, many strategies have been proposed. One is the direct transfer of cytotoxic products into tumor cells (suicide gene therapy). An example of this is the transfer of the herpes simplex tk gene into tumor cells. This gene codes for an enzyme that metabolizes acyclovir and ganciclovir (non-toxic drugs) into lethal metabolites selectively rendering the transfected cells sensitive to treatment with the guanosine analogs ([53]).

A more popular strategy is to invoke enhancement of the immune response. The underlying premise is that the immune system is inherently capable of ridding the body of altered-self cells (tumor cells) ([54] [19]). In fact, this may be a normal response as proposed by immune surveillance theories. Cancers develop because the tumor is able to evade the immune response or the response is not strong enough to effectively deal with the tumor load. Therefore, strategies enhancing any aspect of the immune response may be helpful. These strategies include enhancement of antigen recognition and presentation, cell-mediated cytotoxicity, migration of T cells across endothelial barriers, and establishment and maintenance of long-term protective immunity.

B. The Anti-tumor Immune Response

Cell-mediated immunity, specifically, the action of CD8+ cytotoxic T lymphocytes (CTLs), is felt to be the major effector arm of the response against malignancies ([51] [55] [21]). There are two phases. The first is known as an induction phase, where tumor cells are degraded by inflammatory-type reactions, releasing tumor antigens into the local milieu. These antigens are taken up by APCs (dendritic cells, Langerhans cells, macrophages), processed, and expressed on the cell surface in conjunction with MHC molecules, where antigen can be presented to precursor and effector cells. The second phase is known as the expansion phase. Here, the newly activated T lymphocytes (CD4+ and CD8+) leave the lymph nodes and home to the tumour sites. When they encounter tumor cells, they respond by further proliferation or by exerting their effector function – cytotoxicity or T cell mediated help. Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) has been shown to facilitate the induction phase.

T-cell activation by APCs is a multi-step process that requires at least two signals delivered from the APC to the naïve T-cell ([56], [57], [58], [59]). Signal 1 is delivered via the MHC-Ag complex interacting with the T-cell receptor (TCR) on the T-cell. By itself, this signal is not strong enough to lead to T-cell activation. In fact, delivery of this signal alone may lead to a state of anergy ([59]). Signal 2 is delivered via B7 molecules (B7-1 and B7-2) present on activated APCs that interact with CD28 on T-cells. Following delivery of both signals, T-cell proliferation is induced and T-cells allowed to execute their effector functions (helper and/or cytotoxicity). An intermediate step that links the two signals is the CD40:CD154 interaction (see later section).

In addition to CTLs, other effector cells such as natural killer (NK) cells may also play a role. These CD16+, CD56+, CD4-, CD8- cells are "promiscuous" in that they are not MHC-restricted cytotoxic cells, unlike CD8+ cells that are restricted to Class I MHC targets ([21]). A variable number of melanomas down-regulate MHC Class I expression ([39] [42] [40] [38] [36] [37]); thus, NK cells may play a particularly important role in combating these tumors ([21]).

C. Cancer Vaccines

The classic concept of vaccination is to confer a state of immunity to an individual before that individual encounters the pathogen of note ([60]). This description originated with viral vaccine studies. Tumor vaccines, on the other hand, are administered to patients already harboring tumor, ideally following surgical removal of macroscopic disease. These are therapeutic vaccines, but they may also be prophylactic towards recurrent and/or metastatic tumour. The idea is to prime the immune system against a particular disease by exposing it to antigens specific for that disease. Many sources of antigen exist including attenuated whole cells, cell walls, cell lysates, specific antigens, or non-pathogenic strains of living organisms. Most tumor vaccine strategies utilize whole cells, attenuated by irradiation. [51]

Early work on melanoma vaccine therapy was pioneered by Morton et al ([9]). In 1978, this group developed a vaccine composed of allogeneic melanoma cells mixed with BCG. Unfortunately, this vaccine had little, if any clinical effect. This was attributed to low levels of melanoma-associated antigens on the melanoma cells chosen for the vaccine. In 1984, this group developed a vaccine composed of a mixture of melanoma

cells specifically selected for high levels of antigen expression. A phase II trial commenced in 1985, in which this vaccine was administered to AJCC Stage IIIa and IV melanoma patients. This was a poorly controlled trial as many of these patients also received additional therapy in the form of chemotherapy or biological response modifiers, such as cimetidine, indomethacin, or cyclophosphamide. Overall, patients receiving this vaccine survived significantly longer when compared to historic controls treated by conventional chemotherapy (median survival increased from 7.5 months to 23.1 months) ([9] [10]). The basis for generic vaccines is that many antigens are shared between tumors. They are attractive because they are easier to generate than autologous vaccines.

Autologous vaccines utilize tumor cells harvested from the patient for whom the vaccine is intended. The hypothesis is that the antigens expressed on such vaccines are more specific to the patient. Genetically modified tumor vaccines (GMTVs) represent vaccines employing tumor cells that have undergone genetic modification to increase their immunogenicity ([19]). Examples are vaccines composed of cells genetically transduced with cytokines ([61] [62]), MHC molecules ([36] [37] [63]), and co-stimulatory molecules ([57] [64] [63] [65]).

Many cytokine genes have been investigated for this purpose including the interleukins (1,2,3,4,6,7,10,12), Tumour Necrosis Factor- α (TNF- α), Interferons (IFN- α , - β , - γ), and GM-CSF. The rationale behind this approach is that GMTVs expressing these cytokines will raise local concentrations of a pro-immunity cytokine. This should enhance local antigen presentation and activation of cell mediated immunity. Unlike systemic administration of cytokines which can result in severe toxicity ([23]), toxicity is limited because the high concentrations remain local.

D. Retroviral Vectors

One of the requirements for a gene therapy protocol is an effective delivery system to transfer the gene of interest into target cells. In this matter, there are basically two choices – viral delivery systems and non-viral delivery systems. Included in the latter are chemical methods, such as calcium phosphate transfection, and physical methods, such as liposome-mediated transfer, electroporation, and particle mediated transfer (the gene gun), which are currently being evaluated for use in various gene therapy protocols. Thus far, viral delivery systems have been employed most often and, of these, retroviral systems have been the most popular. Other viral delivery systems include adenoviruses, adeno-associated viruses, vaccinia viruses, and the herpes simplex virus. ([52] [66] [67]).

Retroviruses have been studied for more than 80 years. These organisms have been linked to numerous human diseases, namely neoplasms and the acquired immune deficiency syndrome. They are RNA viruses that are able to infect mammalian cells, integrate their genetic material into the host's genome, and then use the host's cell machinery to replicate. The retroviral genome consists of two single stranded RNA molecules, each consisting of long terminal repeat sequences (LTRs), gag, pol, and env regions, as well as a packaging signal (ψ). LTRs are viral promoters that drive expression of the virally encoded genes, while the gag, pol, and env regions code for viral structural proteins and the enzymes reverse transcriptase, integrase, and protease. The packaging signal is essential to direct the encapsidation of the viral genome. ([67] [66] [52]).

The capacity to accept foreign DNA and the ability to integrate genetic material into host cell genomes make retroviruses natural candidates for gene delivery vectors. The only components necessary for virion formation and genomic integration are the LTRs and ψ . The gag, pol and env genes can be removed rendering the virus replication incompetent. In their place, foreign DNA can be inserted (up to 8kb). A second marker gene is usually also cloned into the vector, such as an antibiotic resistance gene that will allow you to select for cells that have been successfully transduced. These replication incompetent virions are amplified in number by using packaging cell lines that provide the gag, pol, and env genes *in trans*. ([52] [68] [66])

The first retroviral vectors were created in 1981 by three groups working independently ([69] [70] [71]). Subsequently, retroviruses have been used to transfer genes into T cells, hematopoietic progenitor cells, neoplastic cells of hematopoietic lineage, and neoplastic cells of solid tumor origin ([67]). The advantages of retroviral vectors include their relative ease of construction, high efficiency of transfer, and stable integration. However, limitations include the fact that only actively replicating cells are transduced, thus necessitating the need for *ex vivo* approaches that are more time consuming and labor intensive than *in situ* gene delivery systems. Other considerations include the risk of creating replication competent retroviruses, some of which may cause a systemic viremia. Theoretically, such replication competent viruses could capture cellular oncogenes or lead to insertional mutagenesis resulting in deleterious gene disruptions or oncogenesis. There are no published reports that any of these potential side effects have occurred to a clinically significant extent ([67]).

The cDNAs for G-CSF, GM-CSF, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, TNF- α , IFN- α , and IFN- γ are just some of the genes that have been successfully ligated to retroviral vectors, transferred into tumor cells, and, thereafter, shown to increase the immunogenicity of the transduced cells ([61]).

E. SCID/beige Mouse Model

SCID stands for severe combined immunodeficiency. SCID mice are those homozygous for the scid mutation that renders them severely deficient in functional B and T lymphocytes. These immunodeficient mice were a serendipitous discovery by Bosma et al in 1983 ([72]) while quantitating serum immunoglobulin levels in C.B-17 mice. Four out of 7 litter mates lacked the major Ig isotypes and pedigree analysis revealed an autosomal recessive pattern for this mutation. Selective breeding established a colony. These mice have been used as animal models for oncologic diseases, infectious diseases, and AIDS ([73]).

The SCID gene has been mapped to chromosome 16, but the exact location and gene product have yet to be identified ([74] [75]). The genetic defect affects V(D)J recombination of clonotypic receptors. This recombination occurs in an aberrant fashion leading to the arrest of development of B and T cells, thus SCID mice lack functional T and B cells ([75]). However, these mice have normal myeloid cell numbers and function, functional APCs including macrophages and dendritic cells, and normal natural killer cell activity ([76]). These mice are also deficient in DNA repair mechanisms as evidenced by their extreme sensitivity to ionizing radiation ([74]).

It is a concern to researchers that up to 23% of SCID animals are "leaky" i.e. they are not completely deficient in T and/or B cells ([74]). Non-leaky strains have been developed. One such strain is the SCID/beige strain. These mice were generated by crossbreeding with mice homozygous for the beige mutation, which renders such mice deficient in NK cells. SCID/bg mice, therefore, are deficient in functional T, B, and NK cells and have a lower rate of leakiness (~3%) ([73]).

An exciting development has been the creation of humanized-SCID chimeras. McCune et al transplanted human fetal lymphoid tissue and cells into SCID mice leading to immune reconstitution ([77]), while Mosier et al demonstrated that chimeras could also be created by intraperitoneal injection of human peripheral blood lymphocytes into SCID mice ([78]). The latter strategy has become the more popular one given the technical and ethical constraints with the former. This system has been used successfully to study melanoma ([76] [79]), as well as other tumours.

The exact nature of how Hu-PBL-SCID chimeras develop remains to be fully elucidated. Tary-Lehman et al have proposed the following mechanism ([80]). Following intraperitoneal injection, all of the transplanted cell lineages required for a human immune response survive for 10 to 20 days. Thereafter, the only T cells that persist are those reactive to mouse antigens. They have the phenotype of mature/activated memory cells (CD45Ro). Antigens introduced by the tumor vaccine (if given within 20 days of reconstitution) can also activate T cells. Activated T cells disseminate and settle in the liver, spleen, and lung by the 4th week. Peak chimerism occurs between 4-8 weeks post-reconstitution. Thereafter, T cell numbers decrease and human T cells are no longer detectable by the 5th month. The nature of B cell

reconstitution remains unresolved. B cells occur in much lower numbers (4%) and do not appear to be antigen dependent.

The possibility of graft versus host disease complicates this model. This remains a controversial area as some investigators claim that this does not occur ([78] [80]), while others have documented that it does occur ([81]). It is proposed that significant graft versus host disease does not occur because a second signal is not provided and these cells are rendered anergic ([80]).

III. Immunomodulatory Molecules

The molecules of interest include two cytokines (granulocyte macrophage-colony stimulating factor (GM-CSF) and Interleukin-12 (IL-12)) and two cell surface molecules (B7-2 and CD154 (CD40L)).

A. GM-CSF

GM-CSF is a cytokine that was originally described as one of a group of hematopoietic growth factors ([82]). In particular, GM-CSF plays an important role in the maturation and function of specialized antigen presenting cells (macrophages, dendritic cells, and Langerhans cells). Antigen-presentation is a crucial aspect of any immune response, including cell-mediated immunity.

Perhaps the strongest evidence of the importance of GM-CSF was provided by Dranoff et al in 1993 ([83] [84]). The seminal study involved the creation of various tumor vaccines by retroviral transduction of tumor cells with various cytokine genes (IL-1, IL-2, IL-4, IL-5, IL-6, IFN- γ , ICAM, CD2, TNF- α , and GM-CSF). These vaccines were tested on various murine tumour models including colon cancer (CT-26),

fibrosarcoma (CMS-5, WP-4), renal cancer (RENCA), Lewis lung carcinoma, and malignant melanoma (B16). In all models, GM-CSF had the most potent anti-tumour response and this activity was dependent upon the presence of both CD4+ and CD8+ cells. Ultimately, a total of 29 cytokines have been evaluated with this system and GM-CSF remains the most potent anti-tumor cytokine ([84]).

Other lines of evidence include a study by Armstrong et al, which evaluated the effect of GM-CSF on the growth of a murine melanoma (HFH18) ([85]). They generated a melanoma-derived vaccine by transducing melanoma cells with the cDNA for GM-CSF by electroporation and then demonstrated that following administration of this vaccine, melanoma growth was completely inhibited in most cases or resulted in much smaller tumors, when compared to non-vaccinated controls. They also demonstrated a dose-response relationship, as those clones that produced higher amounts of GM-CSF had more of an effect than those clones producing lower levels. They did similar experiments with IL-6 and also found that this strategy inhibited tumor growth, however, the GM-CSF effect was much more dramatic.

In addition to local tumor regression, systemic immunity is also generated. In another murine melanoma model, Mahvi et al ([86]) demonstrated that vaccination with GM-CSF gene-transduced tumor cells protected a majority (58%) of mice that were subsequently challenged with wild type tumor.

Combination regimens have also been evaluated in murine models. Wakimoto et al ([87]) demonstrated that the GM-CSF response could be augmented with the added use of certain biological response modifiers (BRMs), such as OK432. They further investigated combinations of GM-CSF with various cytokines (IL-1, IL-2, IL-3, IL-4, IL-

6, IL-7, IL-12, TNF- α , TNF- β , MIF, LIF, and G-CSF), and found that vaccines combining GM-CSF with IL-4 and with TNF- α resulted in greater anti-tumor activity than with GM-CSF alone. No significant additional effect was seen with the other cytokines.

Another study demonstrating the efficacy of combination therapy was reported by Parney et al ([44]). A bicistronic vector containing the cDNAs for GM-CSF and the T-cell co-stimulatory molecule B7-2 was successfully used to introduce both genes into cultured human glioblastoma multiforme cells. The vaccine expressing both genes had a stronger anti-tumor effect than when only one of the two genes was expressed. In this regard, there have been suggestions that another function of GM-CSF is to also upregulate B7-1 and B7-2 expression on APCs, thus further enhancing APC ability to activate T-cells ([88] [85]).

Regarding human studies, the first case report of the use of a GM-CSF secreting tumor vaccine involved a 46 year old man with Stage IV melanoma who received three injections of the vaccine created by retroviral transduction of autologous tumor cells. There appeared to be regression of axillary metastases and stabilization of pulmonary metastases. Peritumoural cerebral edema increased (suggesting activity against cerebral metastases). Unfortunately, many visceral metastases (adrenal, pancreatic, and splenic) did not respond at all to the treatment. The overall response was only partial and short-lived. The patient succumbed to melanoma 75 days after the last vaccination. ([89])

A phase I study evaluating the safety of autologous melanoma vaccines was begun in early 1997 by Dranoff et al ([84]). Similar studies are concurrently being

carried out at Johns Hopkins University, The University of Alberta, and in the Netherlands.

A number of local and systemic side effects have been identified. Potential local toxicity includes: pustular eruption, necrotizing vasculitis, erythema, pruritus, recall erythema, general papular rash, and phlebitis. Potential systemic toxicity may include fever, bone pain, malaise, diarrhea, transient liver function abnormalities, leukopenia, leukocytosis, arthralgia, dyspnea, fluid retention, serous effusion, and induction of autoimmune diseases. ([84])

B. Interleukin-12

This molecule represents yet another immunomodulator with inherent anti-tumour properties. Interleukin-12 (IL-12) was discovered in 1986 ([90]) and first described as a factor that could synergize with interleukin-2 (IL-2) in the generation of cytotoxic T lymphocytes. It was initially called cytotoxic lymphocyte maturation factor (CLMF) ([91]). Subsequent work by Kobayashi et al ([92]) identified a 70 KDa glycoprotein that induced IFN- γ production by peripheral blood lymphocytes, resulting in enhanced lymphocyte proliferation and cytotoxicity. This group named their molecule natural killer cell stimulatory factor (NKSF). Cloning of CLMF and NKSF revealed that they were one and the same, and the molecule was renamed IL-12 ([93]).

Structurally, IL-12 is a heterodimer of two subunits linked by a disulfide bond. The smaller subunit is a 35 KDa light chain sometimes referred to as p35, while the larger subunit is a 40 KDa heavy chain named p40. Two separate genes encode the two subunits and both have to be expressed in order to get biological activity. p40 shares

homology with the IL-6 receptor, while p35 shares homology with IL-6 and G-CSF.

([93])

IL-12 is secreted mainly by macrophages, dendritic cells, and neutrophils. B cells and Langerhans cells may express it at low levels, but it is not normally elaborated by T-cells. The IL-12 receptor is a 110 KDa molecule expressed on activated T-cells, NK cells, as well as B cells ([94]). IL-12 is important in the development of a Th₁-type (primarily cell-mediated) response.

A number of *in vitro* studies have been carried out and collectively they suggest that IL-12 has a synergistic role with IL-2 to: (1) enhance allogeneic CTL responses, (2) enhance stimulation of LAK cell activity, (3) enhance IFN- γ production by peripheral blood lymphocytes, (4) induce secretion of IFN- γ and other cytokines by T and NK cells, and (5) enhance proliferation and cytotoxic activity of T and NK cells. ([94])

In vivo studies have generally corroborated the *in vitro* findings. Specifically, IL-12 has been found to (1) augment NK cell lytic activity, (2) induce IFN- γ release by NK and T cells, (3) enhance allogeneic responses, and (4) mediate potent antitumor effects ([95] [93]).

As previously mentioned, the cell-mediated arm of the immune response is of primary importance in anti-tumor immunity. In murine models, both direct intratumoral injection and systemic administration of IL-12 have resulted in an anti-tumor response. These include B16F10 murine malignant melanoma, M5076 reticulum cell sarcoma, RENCA (renal cell adenocarcinoma), MC38 colon adenocarcinoma, and methylcholanthrene-induced sarcomas. Observed effects include inhibition of tumor growth, enhanced survival, and decreased metastases. In the RENCA model, peritumoral

injection of IL-12 had a greater effect than systemic administration ([96]). This suggests that paracrine delivery resulting in high local concentrations is a more promising mode of delivery than is systemic administration. Additionally, IL-12 has been shown to have intrinsic anti-angiogenesis activity, presumably through stimulation of IP-10 production ([97]).

C. B7-2

It is now accepted that at least two signals are required to activate a T cell ([98] [57] [99]). The first signal is the TCR interaction with antigen presented by an APC in the context of the appropriate MHC molecule. The second co-stimulatory signal is provided by the APC to the T-cell (a co-stimulatory molecule expressed on the cell surface which binds to its ligand on the T-cell). Molecules with co-stimulatory properties include the B7 superfamily (B7-1, B7-2, B7-3), CD40, ICAMs, LFA-3, VCAMs, and HSA ([98]).

Without the second signal, T cell anergy results ([59]). This may be a mechanism of developing self-tolerance. Freeman et al suggested that once a T cell receives the first signal, it must commit to either activation or anergy, depending on the presence or absence of the co-stimulatory signals ([100]).

The original B7 molecule (B7-1) was first described by Linsley et al in 1989 ([99]). It was known to have two natural ligands – CD28 and CTLA-4. A second ligand (B7-2) for CD28 and CTLA-4 was discovered in 1993. Subsequently, B7-1 was renamed CD80 and B7-2 was renamed CD86. Human B7-2 is sometimes referred to as B-70. A

third member of the family, named B7-3 has also been discovered, but its function has yet to be determined. ([100])

B7-2 is constitutively expressed on dendritic cells, macrophages, and activated B-cells. B7-1 is expressed on these same cells only following activation. These molecules are not normally expressed on solid tumors ([64]); however, B7 molecules have been found on B-cell lymphomas ([100]). Both bind similarly to CD28 and CTLA-4, with both having approximately 100 fold more affinity for CTLA-4; however, B7-2 dissociates more slowly of the two from CTLA-4 ([56]). The fact that their binding patterns are somewhat similar is surprising since their extracellular domains share only about 26% sequence homology ([100]). The CD28-B7 molecule interaction strengthens the affinity of T-cell-APC binding across the antigen bridge and possibly facilitates signal transduction via the TCR complex. B7-CD28 binding also results signal transduction all its own across the T cell membrane, possibly involving activation of the phosphoinositide 3-kinase ([56]). The entire pathway has yet to be elucidated, but the end result is an increase in IL-2 production and T-cell proliferation ([58]). The role of CTLA-4 remains controversial. One theory is that the role of CTLA-4 is to compete for ligands, thereby terminating the CD28 pathway. However, Linsley has postulated that CTLA-4 binding to B7-1 and/or B7-2 simply potentiates B7-CD28 binding and signal transduction ([99]).

B7-1 and B7-2 differ in that B7-2 (but not B7-1) mRNA is constitutively expressed on non-stimulated B cells, dendritic cells, and monocytes. Freeman thus proposes that B7-2, not B7-1, is the molecule that provides the early critical co-stimulatory signal that leads to T cell activation rather than anergy. B7-1 appears on the cell surface later (24-48 hours after activation), thus, it may be responsible for providing

the signal that results in T-cell clonal expansion and maintenance of the T-cell response.

([100])

A number of studies have demonstrated that transfection of tumor cells with either B7-1 or B7-2 can increase the immunogenicity of tumors ([101] [102] [63] [103]).

Martin-Foncheca et al further demonstrated in relatively non-immunogenic tumors (in this case melanoma and adenocarcinoma) that B7-2 provided a stronger stimulus to increase tumor immunogenicity than B7-1 ([102]).

D. CD154

CD154 is a 33 kDa transmembrane protein found on the cell surfaces of activated CD4⁺ T-helper cells ([104]), mast cells, eosinophils, and basophils ([105]). It is the natural ligand for CD40, which is a 40 kDa transmembrane protein found on antigen presenting cells (APCs), such as dendritic cells (DC) ([106] [107]), macrophages ([108]), monocytes ([109]), and B-cells ([106]), as well as vascular endothelial cells and thymic epithelial cells ([110]). This receptor-ligand pairing is related to the tumour necrosis factor receptor (TNFR)-tumour necrosis factor family of receptor-ligand pairings ([111]). Other important members of this family include FAS:FAS-Ligand CD27:CD70, TRAIL: TRAIL-R1/2, and CD30-CD30-Ligand ([106]).

The importance of the CD40:CD154 interaction was first noted in B-cells. A syndrome referred to as X-linked Hyper IgM syndrome was found to be associated with a defective CD154 gene ([108]). In this condition, there is lack of germinal centre formation, lack of class switching of immunoglobulin isotypes, and an inability to establish B-cell memory. Affected individuals were susceptible to recurrent upper

respiratory tract infections, cryptosporidial diarrhea, and pneumocystis carinii pneumonia because of the defect in humoral immunity. The subsequent discovery of a defective CD154 gene led to the postulate that the humoral defect was secondary to lack of T cell help that is necessary to allow B-cell differentiation and proper function. Further evidence to support this notion comes from work done with CD40- and CD154- knockout mice ([106] [112]). Both sets of mice show similar defects in humoral immunity. In addition, CD154 knockouts demonstrate an increased susceptibility to Leishmaniasis, indicating that there is also a defect in T-cell responses (T_{H1} in particular). This T_{H1} defect can be corrected with the administration of IL-12 or soluble CD154 ([113]).

The CD40:CD154 receptor-ligand pair is believed to play a key role in antigen presenting cell (APC) interactions with T-cells in a pathway that eventually results in activation of CD8⁺ cytotoxic T-lymphocytes (CTLs) ([114] [115] [116] [117]). This pathway begins with antigen being recognized in the periphery by APCs. Antigen is captured, processed, and eventually presented on the surface of antigen presenting cells in the context of Class I and Class II HLA molecules. Following migration to a lymph node, the APC encounters naïve T-cells of appropriate antigenic specificity. Previous theories proposed that a simultaneous 3-cell interaction occurred between the APC, a CD4⁺ T-helper cell, and a CD8⁺ T-killer cell ([118]). The APC activates both cell types through two signals – signal one is the interaction between HLA-Ag with the T-cell receptor (TCR) and signal two is the interaction between B7 molecules on the APC with CD28 on the T-cell. Both signals must be provided in order to establish full activation of T-cells. It was also theorized that once activated, the CD4⁺ T-helper cell could provide “help,” in the form of cytokine secretion (IL-2), to assist in the activation of CD8⁺ T-

killer cells. Ridge et al ([119]) argued that a simultaneous 3-cell interaction between 3 migratory cell types that also depends on sharing of antigenic specificity would be a low probability interaction. They have proposed an alternative theory that involves the CD40:CD154 interaction as an intermediary step. This theory (termed the "Licensing theory") proposes that the initial APC-T-cell interaction occurs between the APC and a CD4⁺ T-cell. Signal one is delivered, as described above. The CD4⁺ T-cell responds by transiently upregulating expression of CD154 on its cell surface. Roy et al ([104]) et al demonstrated that expression is transient, first detected at 4 hours, peaking by 8 hours, and all but gone by 48 hours. When present, CD154 can bind to CD40 constitutively expressed on APC cell surfaces. Ligation of CD40 effectively "primes" the APC. "Priming" entails upregulation of co-stimulatory molecules like B7-1 (CD80), B7-2 (CD86), ICAM, and others. APC production of pro-inflammatory cytokines like IL-12, IL-1, and TNF α are also increased. Furthermore, it has been demonstrated that CD40 ligation of DC and monocytes rescues these cells from apoptosis ([120] [121]). A "primed" APC is more effective at delivering co-stimulatory signals and its cytokine profile promotes a pro-inflammatory, or T_H1, response. Such a cell would be very effective in activating subsequent CD8⁺ T-killer cells that it happens to encounter. It is argued that a sequential 2-cell interaction such as this is more probable than the simultaneous 3-cell interaction previously proposed ([118] [119]).

Support for this theory is as follows. Bennett et al ([116]) demonstrated that mice lacking T-helper cells were unable to mount a T-killer response when exposed to appropriate antigens. This effect can be overcome with the administration of stimulating anti-CD40 antibodies. In effect, these stimulating antibodies take on the role of T-helper

cells by priming APCs so that they can provide co-stimulatory signals to, and thus, activate T-killer cells. Schoenberger et al ([117]) demonstrated a similar effect in mice depleted of CD4⁺ T-cells. In addition, they demonstrated that normal activation of T-killer cells could be blocked by anti-CD154 blocking antibodies. A similar effect is seen with the use of blocking anti-CD40 antibodies. Ridge et al ([119]) performed a series of experiments in which they demonstrated that DC were not able to stimulate T-killer responses unless they were first primed by T-helper cells or with stimulating anti-CD40 antibodies. They also demonstrated that these interactions did not need to be simultaneous in order to achieve the desired response.

The CD40-CD154 interaction in malignancies requires further investigation. Previous studies demonstrated that the use of certain tumour vaccines is dependent on this interaction. In CD40 knockout mice and in mice treated with blocking anti-CD154 antibodies, tumour vaccines that were effective in wild-type mice lost their protective effect ([122]).

In terms of tumour expression of these two molecules, CD40 has been detected on the surfaces of melanoma and urinary bladder tumours ([123]). van den Oord et al ([124]) looked at paraffin sections of excised melanoma specimens and found that 59% of these samples expressed CD40. Furthermore, in a subset of these CD40⁺ tumours, 44% were also CD154⁺. The exact significance of these findings is not entirely clear; however, retrospective analysis of patient charts revealed that CD40⁺CD154⁺ tumours tended to do worse clinically.

E. Rationale for Combination Therapy

The preceding sections provided an overview of immune modulating molecules, which are primarily involved in the cell-mediated immune response against malignancies. The 4 molecules described are known to upregulate the cell-mediated response pathways, but at different points along the pathway. Utilizing a combination approach it would appear that upregulation of this system at more than one point ultimately leads to a stronger cell-mediated response. Furthermore, given that a variety of cancer-induced immune evasion mechanisms exist and that any given tumour is a heterogeneous group of cells, it is reasonable to expect that more than one immunosuppressive mechanism may be at work. Thus, adoption of a combined immune gene strategy appears to have greater potential in enhancing cell-mediated immunity against cancers such as melanoma.

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Chapter 2

Retroviral-Based Combined Immune Gene Therapy for Human Malignant Melanoma in a Hu-PBL-SCID/bg Mouse Model

Introduction

Malignant melanoma is a neoplasm of increasing significance. This once uncommon malignancy is now the 7th most common cancer in North America ([1]). Most worrisome is that melanoma is a tumour with a strong propensity to metastasize, and, once metastatic, carries a dismal prognosis. Five-year survival for metastatic disease (Stage IV) is about 5% with a median survival of 6 to 8 months ([2]). Traditionally, therapy for advanced disease has involved some form of chemotherapy. Unfortunately, response rates have been quite low with very few patients experiencing long-term complete responses and in many cases, toxicity can be very significant. ([3] [4] [5])

Immune-based strategies have recently been proposed as a possible alternative to chemotherapy ([6]). Melanoma is an attractive target for such therapies as it appears to be an inherently immunogenic tumour. There have been many case reports of spontaneous regression of lesions, which is explained by some as representing immune eradication of tumours ([7]). This is supported by the findings of intense lymphocytic infiltration within areas of regression ([8]). Furthermore, numerous melanoma associated tumour antigens have been identified, and some of these serve as recognizable targets for both humoral and cell-mediated immune responses in *in vitro* systems ([9] [10]). There have also been encouraging clinical studies including the work of Morton et al in the 1970s, in which intralesional injection of bCG (bacille Calmette-Guerin) was associated with lesional regression ([8]). Rosenberg's work with interleukin (IL)-2 in adoptive transfer strategies in the 1980s ([11] [12] [13]) has spawned numerous clinical trials involving some form of biologic therapy. More recently, Kirkwood et al demonstrated in a prospective randomized fashion that interferon (IFN)-alpha2b given systemically

improved the survival of advanced stage melanoma patients ([14]). To date, this is the only study which provides level 1 evidence of some form of adjuvant therapy improving survival. However, even in the case of IFN- α 2b, improvement in survival was modest and toxicity was still an issue. Currently, there are no less than 79 registered clinical trials in the United States alone looking at adjuvant therapies, most involving some form of immune therapy (www.NCI.com).

In the following study, we explore the potential of three molecules to upregulate the immune response towards human malignant melanoma. Genetically modified tumour vaccines (GMTVs) were created by retroviral transduction of cultured melanoma cells, generating cells that express granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-12, and/or B7-2 (CD86). These three molecules are known to have immunostimulatory properties.

GM-CSF is a cytokine that is known to promote the growth of and enhance the function of antigen presenting cells (APCs) ([15]). Dranoff et al has demonstrated in a murine melanoma system, that of a panel of 29 immunomodulating factors, GM-CSF had the most potent tumour-inhibitory effect ([16] [17]). IL-12 is another cytokine with anti-tumour properties ([18]). Its major roles are to promote the proliferative capacity and cytotoxicity of cytotoxic T-cells (CTLs) and natural killer (NK) cells ([19] [18] [20]). Our other molecule of interest is B7-2, which is naturally occurring on APCs and plays an important role in the delivery of co-stimulatory signals in the process of T-cell activation ([21] [22] [21]). This molecule also appears to have anti-tumour properties ([23]).

Previous work in our laboratory demonstrated that in a chimeric Hu-PBL-SCID/bg mouse-human glioblastoma multiforme model, therapeutic vaccination with a GMTV expressing both GM-CSF and B7-2 resulted in regression and then eradication of transplanted glioblastoma multiforme ([24]). This study is an attempt to demonstrate a similar response with human melanoma. Specifically, our hypothesis is that in a chimeric Hu-PBL-SCID/bg mouse-human melanoma model, therapeutic vaccination with GMTVs expressing GM-CSF, IL-12, and/or B7-2 will inhibit the growth of transplanted human melanoma. Our other major premise is that a combination approach will be more effective than monotherapy.

Methods

Melanoma Culture Technique

Melanoma cultures were established using a modification of a protocol designed for establishing glioblastoma cultures ([24]). Briefly, fresh samples of excised tumour were obtained at the time of operation and expeditiously transported to our lab for processing within 24 hours. The sample was minced into 3 x 3 x 3 mm samples and digested in Hank's Balanced Saline solution (HBSS; GibcoBRL) containing 2 mg/mL Collagenase II (GibcoBRL,) and 0.1 mg/ml Hyaluronidase (Roche; Laval, Quebec) with gentle stirring at room temperature for 1 to 3 hours. The resulting suspension was filtered through a fine mesh sieve and centrifuged over a Ficoll-Paque (Pharmacia Biotech, Baie d'Urfe, Quebec) density gradient at 640 x g for 30 minutes. An interface layer containing predominantly melanoma cells was harvested, washed twice in HBSS, and then plated onto T-25 tissue culture flasks (Corning, Acton, Massachusetts). All melanoma cells

were grown at 37°C, 5% CO₂ in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 I.U./ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Supplemented media is hereafter referred to as complete RPMI. (Media and supplements were all purchased from GibcoBRL). Cells were fed and passaged as necessary.

Retroviral Vectors

The following retroviral constructs were used: pLSN-B70, pLSN-GM1, pLSN-IL12, and pLSN-BG9. Figure 2-1 shows diagrammatic representations of these vectors. All are based on the retroviral vector pLSN, which was derived from modified Moloney murine leukemia virus ([25]). pLSN-B70 contains the gene for B7-2 while pLSN-GM1 contains the gene for granulocyte macrophage-colony stimulating factor (GM-CSF). pLSN-IL12 and pLSN-BG9 are both bicistronic vectors, each containing 2 genes in sequence and separated by an internal ribosome entry site (IRES) sequence. In the pLSN-IL12 vector, the gene encoding the p40 subunit of IL12 is found upstream to the gene encoding the p35 subunit. The pLSN-BG9 vector contains the B7-2 gene upstream to the GM-CSF gene. All inserts were under control of the retroviral LTR promoter. All vectors also contained a neomycin resistance gene driven by an SV40 promoter.

These replication incompetent vectors lack *gag*, *pol*, and *env* genes. Infectious virions were generated using PA317 and PG13 packaging cell lines (both from American Type Culture Collection (ATCC)) which provide the necessary genes for replication ([26]). PA317 cells were grown in T-25 flasks in complete Dulbecco's modified Eagle's media (DMEM; GibcoBRL) (supplemented with FBS, penicillin, streptomycin, and L-glutamine as for RPMI-1640). When the cells reached 70% confluence, they were

transfected with 10 μ g of retroviral plasmid DNA using Calcium Chloride precipitation. A mock-transfected flask of PA317 cells served as a negative control. After an overnight incubation with the DNA-precipitate at 37°C, 5% CO₂, the DNA-containing media was removed and replaced with complete DMEM containing up to 1000 μ g/ml of G418 resulting in the demise of untransfected cells. When all of the control cells had died, selection was considered complete. Fresh DMEM was provided and the transfected PA317 cells allowed to grow to 70% confluence. Supernatant containing infectious, replication-incompetent retrovirus was harvested and divided into 0.5 ml aliquots and frozen at -70°C or immediately used to transduce the second packaging cell line, PG13, growing in T-25 flasks in complete DMEM. Upon reaching 70% confluence, PG13 cells were incubated with 0.5 ml of thawed infectious virus harvested from PA317 supernatants, 1 ml of DMEM, and 4 μ g/ml of polybrene for 3 hours at 37°C, 5% CO₂. 2.5 ml of fresh DMEM was added and the mixture incubated overnight. Again, a mock-transduction flask containing no retrovirus served as a control. Antibiotic selection followed, as above. Virus-containing supernatant was harvested, divided into 0.5 ml aliquots, and frozen at -70°C for later use.

Retroviral Transduction of Cultured Melanoma Cells

Wild-type (WT) cells growing in T-25 culture flasks were transduced when they reached 70% confluence. Old media was aspirated and replaced with 1 ml of fresh complete RPMI-1640, 0.5 ml of thawed virus from PG13 cells, and 4 μ g/ml of polybrene. This was incubated at 37°C, 5% CO₂ for 3 hours, then topped up with 2.5 ml of fresh complete media and incubated overnight. A mock-transduction flask containing no

retrovirus served as a negative control. Antibiotic selection with RPMI-1640 with G418 (250-1000 μ g/ml) followed as above. When selection was complete, fresh RPMI-1640 (less G418) was added and the cells allowed to grow to adequate numbers.

Flow Cytometry Analysis of Wild-Type and Transduced Melanoma Cells

Melanoma cells growing in T-150 culture flasks were detached and harvested with 0.04% EDTA in Phosphate-buffered saline (PBS). After centrifugation at 1000 rpm, the supernatant was discarded and the pellet resuspended in immunofluorescence buffer (IFB; 2% FBS, 0.02% sodium azide in PBS) and transferred to 1.5-ml tubes (Eppendorf) in 200 μ l aliquots. Primary antibody was added to the tubes at 1:200 dilutions. After a 1 hour incubation at 4°C, the fluorescently-tagged cells were washed 3 times with IFB, then resuspended in 500 μ l of 1% formaldehyde in PBS. If the cells were first labeled with unconjugated antibodies, an anti-Ig FITC-conjugated secondary antibody was added and the cells incubated for 1 more hour at 4°C, washed 3 times with IFB, then resuspended in 500 μ l of 1% formaldehyde in PBS. The cells were then analyzed on FACScan (Becton-Dickinson).

The following fluorescence-conjugated antibodies were used: anti-human Class I HLA-FITC (Caltag; Hornby, Ontario), anti-human HLA-DR-FITC (Caltag), anti-human CD80-FITC (ID Labs), and anti-human CD86-RPE (ID Labs). The unconjugated antibodies used were anti-human Fas (PharMingen) and anti-human Fas Ligand (PharMingen). Anti-mouse IgG-FITC (Sigma) was used as the secondary antibody. Isotype-matched antibodies were also used as negative controls.

ELISA Analysis of Wild-Type and Transduced Melanoma Cells

Supernatant from selected WT and transduced cells were analyzed for expression of IL12 and GM-CSF. Supernatant above attached cells was collected and centrifuged to remove debris. Samples were assayed with commercial kits for IL12 and GM-CSF (R&D Systems; Minneapolis, Minnesota) as per manufacturers instructions or were sent to an independent lab (Dr. J. McElhaney, The University of Alberta), which provided an ELISA service.

Stimulation of Peripheral Blood Mononuclear Cells with Cultured Melanoma Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat samples obtained from Canadian Blood Services (formerly known as the Canadian Red Cross) or from blood obtained from selected melanoma patients. Buffy coat or peripheral blood was first diluted 1:1 with HBSS and then centrifuged over a Ficoll-Paque (Pharmacia Biotech) density gradient at $640 \times g$ for 30 minutes. An interface layer containing predominantly PBMCs was collected, washed twice in HBSS, counted, and then resuspended in complete RPMI-1640 at a concentration of $1-2 \times 10^7$ cells/ml. One ml aliquots were frozen at -70°C for later use.

WT and transduced melanoma cells were used as stimulators in mixed melanoma-PBMC cultures. Melanoma cells were plated onto 24-well tissue culture plates (Corning) at a density of 5×10^4 cells/well and then irradiated with 20,000 rads to halt further replication. Previously frozen PBMCs were thawed and resuspended at a concentration of 1×10^6 cells/ml of complete RPMI-1640 supplemented with 20 units/ml interleukin 2 (IL2). Media overlying the irradiated melanoma cells was removed and replaced with 1

ml of the PBMC suspension. The mixed culture was incubated at 37°C, 5% CO₂ for 7 days, after which the PBMCs were harvested, counted by trypan blue exclusion, then resuspended in fresh RPMI-1640 with 20 units/ml IL2, and re-plated over fresh stimulator cells for another 7 days. At the end of the second round of stimulation, the PBMCs were harvested, counted, analyzed by flow cytometry, and put through a ⁵¹Chromium-release cytotoxicity assay. Flow cytometry looked at expression of lymphocyte markers using the following antibodies: anti-human CD3-FITC/CD56-RPE, anti-human CD4-FITC, and anti-human CD8-RPE (all from PharMingen).

Mouse Experiments

Six to eight week old SCID/bg mice were obtained from Taconic, Charles River, or local stock. Mice in any single experiment were all from the same source, though different experiments may have used mice from different sources. All mice were housed in virus and antigen free conditions. Mouse rations were autoclaved and cotrimoxazole was provided in drinking water.

Following a one-week acclimatization period, mice were injected subcutaneously in one or both flanks (while under metofane anaesthesia) with $1-2.5 \times 10^6$ melanoma cells in 0.25 ml of HBSS. The cell suspensions used for injections were prepared from cell cultures by first lifting the cells off of the culture flasks with trypsin, then making appropriate dilutions in HBSS. Mice were monitored every 3-7 days. By day 14, the majority of mice had small, but palpable tumours. At this point, they were given an intraperitoneal injection of $1-2 \times 10^7$ peripheral blood mononuclear cells isolated from random donors, as previously described. Within 24-72 hours of immune reconstitution, 5

$\times 10^5$ irradiated vaccine cells in 0.25 ml of HBSS were injected intraperitoneally. Mice were monitored and tumours measured every 3-7 days. Two weeks following reconstitution, tail bleeds were performed on all mice and this blood was tested for the presence of human immunoglobulin by ELISA. All mice expressing fewer than $1\mu\text{g/ml}$ were excluded from statistical analyses.

Modifications were made in individual experiments. These are explained more thoroughly in the results section.

Statistical Analyses

Statistical analyses of differences between therapeutic groups and appropriate controls were performed with analysis of variance (ANOVA) testing (Dunnett's or Student-Newman-Keuls post-hoc analysis).

Results

Melanoma Cell Cultures

Between May, 1997 and June, 1999, 31 samples of resected melanomas were obtained by our lab and processed. 22 samples were from male patients and 9 were from female patients.

Using the technique described in the Methods section, 25 melanoma cultures were successfully raised (80.6%), meaning that at least 3 culture passages occurred and enough cells raised for freezing. The remaining 6 tumours were either lost to fungal or bacterial contamination, or they simply did not survive in the culture conditions and the flasks

became overgrown with fibroblasts. Additionally, a commercially available cell line SKMel-28 was purchased from ATCC and maintained in culture in our lab.

Generally, melanoma identity was confirmed by morphologic criteria. Cultured melanoma cells were spindle-shaped and shiny under a phase contrast microscope. They exhibited no contact inhibition and typically grew in sheets of cells. They did not develop a whorling pattern, as was often seen in fibroblast cultures. Melanoma associated tumour markers (Melan-A, HMB45, and S-100) were assayed for in 12 cases – Ed137.Mel, Ed141.Mel, Ed141A1.Mel, Ed153.Mel, Ed156.Mel, Ed157.Mel, Ed162.Mel, Ed165.Mel, Ed169.Mel, Ed176.Mel, Ed178.Mel, and SKMel-28 (Table 2-1). Five (Ed141.Mel, Ed141A1.Mel, Ed153.Mel, Ed162.Mel, and SKMel-28) bore at least 2 of these antigens and were considered true melanoma cultures. Four tumours (Ed141.Mel, Ed141A1.Mel, Ed162.Mel, Ed176.Mel, and SKMel28) were also found to be tumorigenic in SCID/bg mice, providing further confirmation of their malignant origins.

Cell surface expression of Class I HLA, Class II HLA, FAS, FAS-Ligand, CD80 (B7-1), and CD86 (B7-2) were checked in 11 cultured tumours (Table 2-2). 36.4% of the cultured cells had undetectable expression of Class I HLA by flow cytometry. 9/11 (81.8%) had undetectable levels of Class II HLA. All 11 of the tumours expressed FAS and only 1/11 (9.1%) expressed FAS-Ligand. None of the wild type cells expressed CD86, but 7/11 (63.6%) had weak expression of CD80.

Retroviral Transduction of Melanoma Cells with B7-2, GM-CSF, and IL-12

The results of CD86 expression following retroviral transduction with either pLSN B70 or pLSN BG9 are shown in Table 2-3. Both retroviral vectors were successful

at transferring the CD86 gene in all cases. Representative flow cytometry histograms (from Ed194.Mel) are depicted in Fig. 2-2.

GM-CSF levels as determined by ELISA are found in Table 2-4 for melanoma cells transduced with either the pLSN GM1 vector or the pLSN BG9 vector. Expression of GM-CSF following transduction with pLSN BG9 is about 100-fold lower than following transduction with pLSN GM1.

IL-12 levels following transduction with pLSN IL12 are found in Table 2-5. In all cases, IL-12 levels are very low.

Stimulation of Peripheral Blood Mononuclear Cells

The wild-type and transduced forms of the melanoma cell line Ed141.Mel were used to stimulate allogeneic peripheral blood mononuclear cells (PBMCs). Proliferation was significantly enhanced after 14 days of stimulation with Ed141.Mel WT (ANOVA, Dunnett's comparison, $p < 0.05$), Ed141.Mel pLSN-GM1 ($p < 0.05$), Ed141.Mel pLSN-BG9 ($p < 0.01$), and a 1:1 combination of Ed141.Mel pLSN-BG9 and Ed141.Mel pLSN-IL12 ($p < 0.05$) when compared to no stimulation (Fig. 2-3). Ed141.Mel pLSN IL12 induced a slightly increased proliferative response, but not to a statistically significant extent. In the ^{51}Cr -release assay depicted in Figure 2-4, only the Ed141.Mel pLSN BG9 stimulated cells demonstrated statistically greater cytotoxicity when compared to non-stimulated PBMCs (ANOVA, Dunnett's comparison, $p < 0.05$).

Mouse Experiments

Five separate vaccination experiments were carried out in the Hu-PBL-SCID/bg mouse-human melanoma model. The first experiment was carried out using Ed141A1.Mel. The A1 designation refers to a tumour that had previously been grown in an non-reconstituted SCID/bg mouse and re-established back into tissue culture. This was initially done to ensure that we were working with a tumour that grew well in SCID/bg mice. Figure 2-5 summarizes the results of treatment with vaccines expressing IL-12 (Ed141.Mel pLSN IL12), CD86 and GM-CSF (Ed141.Mel pLSN BG9), all three molecules (1:1 combination of each vaccine). The controls used were animals given irradiated wild-type Ed141.Mel and non-treated animals. All vaccinated groups had significantly smaller tumours ($p < 0.05$) than the non-treated animals from day 22 until the experimental end-point. However, no significant differences were seen between the vaccination groups, including those given the wild-type vaccine, though there was a trend in the animals treated with the BG9 vaccine to harbor smaller tumours.

In order to address the effects of antigenic drift, inadequate vaccination, and the independent effects of reconstitution, the experiment was repeated in a modified fashion. In this second experiment, the tumour used was Ed141.Mel, which had not been passaged through a SCID/bg mouse. Additionally, two vaccinations were given (days 14 and 26), and control animals consisted of reconstituted, but non-vaccinated animals. The effects of therapy with vaccines expressing GM-CSF alone (Ed141.Mel pLSN GM1), CD86 alone (Ed141.Mel pLSN B70), GM-CSF and CD86 (Ed141.Mel pLSN BG9), GM-CSF, CD86, and IL-12 (a 1:1 mix of Ed141.Mel pLSN BG9 and Ed141.Mel pLSN IL-12) and a wild-type vaccine are summarized in Figure 2-6. No significant differences were seen

between the vaccination groups or between vaccinated animals and the non-vaccinated controls.

The third *in vivo* experiment was performed using Ed162.Mel. This differed from the previous tumours in that Ed162.Mel expressed Class I MHC; thus, we theorized that this would be more susceptible to a T-cell-mediated response. Also, in order to ensure that the vaccine cells remained antigenically similar to the inoculated tumour, untransduced tumour cells were added to the transduced vaccines in a 1:10 ratio. The results are shown in Figure 2-7. Again, there were no significant differences between the groups.

The final two experiments were performed using Ed176.Mel. All of the previously described modifications were again performed. Additionally, only one flank was inoculated with tumour in an attempt to reduce the tumour burden. Figure 2-8 shows that in an allogeneic system, there were no significant differences between vaccinated animals and the unvaccinated controls. Finally, in a much smaller autologous system in which PBMCs were procured from the patient from whom Ed176.Mel was raised and used to reconstitute the animals. In this experiment, no significant growth inhibition was noted with use of the autologous BG9 vaccine (Figure 2-9).

Discussion

The development of melanoma cell cultures has led to an increased understanding of melanoma immunobiology. Our study of cell surface expression of various molecules adds further information. FAS expression was consistently detected on all samples tested. The corollary to this finding is that these cells could be susceptible to FAS-

mediated killing. Furthermore, only 1/11 cell types tested expressed FAS-Ligand. This is important because some authors have suggested that tumour-expression of FAS-Ligand may be immunosuppressive ([27] [28]). This does not appear to be likely in the melanoma cells tested. A secreted form of FAS-Ligand may exist, but this was not assayed for. Additional information garnered from this study is that a significant proportion (36.4%) had non-detectable levels of Class I HLA molecules. This is consistent with published studies that suggest that this is a form of immune evasion ([29] [30] [31]). Such cells would be difficult targets for cytotoxic T-cells, but they should still be susceptible to natural killer (NK) cell actions [6]). Some of the tumours had weak expression of CD80 and none had constitutive expression of CD86. Lack of significant expression of co-stimulatory molecules may be one reason why these cells aren't more immunogenic ([32] [33] [23]).

Use of cultured cells does have limitations ([34]). Foremost is that clonal selection occurs and the cells grown in culture may not be all that representative of the original *in situ* tumour. The melanoma associated antigens and other cell surface determinants may be very different in cultured cells. Furthermore, the longer cells stay in culture the greater these differences become, as even malignant cells can undergo further *in vitro* transformation.

Retroviral vectors were chosen to transfer our genes of interest into melanoma cells primarily because this technique allows stable gene transfer. The efficiency of retroviral transduction is quite low with only about 5-10% of cells successfully incorporating the provirus. However, because the provirus also contains an antibiotic resistance gene, successfully transduced cells can be isolated from a background of

untransduced cells by screening for antibiotic resistance. All surviving cells carry our genes of interest and large populations can be grown up for further study; however, even though all resulting cells theoretically carry the transduced gene(s), not every cell expresses the gene(s) as would be predicted. In part, this may be due to incomplete antibiotic selection and contamination with non-transduced cells, which may have a survival advantage. It has also been suggested that the introduction of more than one gene, each driven by separate promoters as is the case with our vectors, leads to suppression of one or both of the genes ([35]). A possible mechanism is through methylation of cytosine residues or even through mutations and deletions ([36]). This point is most evident in our bicistronic constructs pLSN BG9 and pLSN IL12. GM-CSF expression by cells transduced with the pLSN BG9 vector is 100 fold lower than that seen in similar cells transduced with pLSNGM1, a monocistronic construct. Similarly, IL-12 levels per 10^6 cells/24 hours are in the picogram range rather than the expected nanogram range. Though we did not specifically assay for each subunit of IL-12, we suspect that there is suppression of expression of the downstream p35 subunit, thus, limiting expression of intact IL-12.

In the peripheral blood mononuclear cell (PBMC) stimulation experiments, we hoped to demonstrate that our genetically modified tumour vaccines could be used as stimulators to generate a cell-mediated immune response. This type of response is felt to be key in immune eradication of tumours ([37] [38] [6]). PBMCs are a heterogeneous group of immune cells, comprised primarily of T-lymphocytes and with relatively smaller populations of B-lymphocytes, natural killer cells, and antigen presenting cells. All the elements are present to generate a cell-mediated response with appropriate stimulation.

Our 3 genes of interest each have the potential to cause upregulation of an immune response at different points in a common pathway. GM-CSF may act on antigen presenting cells, resulting in upregulation of co-stimulatory molecule expression ([39]). IL-12, provided in high local concentrations by vaccine cells, could potentiate activation of nearby cytotoxic T-killer cells and natural killer cells ([18]). Finally, by expressing CD86 (B7-2) molecules on their cell surfaces, vaccine cells may enhance the immune attack against itself by providing signal 2 to CD8⁺ cytotoxic T-cells with the appropriate antigenic specificity ([23] [32]). We found that concurrent provision of both GM-CSF and CD86 (B7-2) produced significantly higher lymphocyte proliferation and cytotoxic potential. Our results further suggest that addition of the IL-12 vaccine to our cocktail of immune stimulating molecules offered no beneficial immune enhancement. In fact, IL-12 appears to have inhibited the immune response. This may be secondary to the inhibitory effects of excess amounts of the p40 subunit of IL-12, which we think is occurring with our bicistronic pLSN IL12 construct ([35]).

The promising effects of the BG9 vaccine in our *in vitro* studies did not carry forward to our *in vivo* mouse studies. There are published studies that do demonstrate tumour inhibition by similar molecules in similar models. For example, Dranoff et al demonstrated the efficacy of GM-CSF in treatment of murine melanoma ([16]), Baskar et al demonstrated that mouse sarcomas could be made more immunogenic following transduction with B7 molecules ([33]), and Bramson et al demonstrated the tumour inhibitory effects of IL-12 using a murine breast cancer model ([40]). Our experiments differed in that human tumours, not murine, were used. Previous work in our lab ([24]) demonstrated quite dramatic tumour growth inhibition of human glioblastoma multiforme

following vaccination with the BG9 vaccine using a similar mouse model. In contrast, 5 separate experiments using cultured melanoma obtained from three different patients failed to show that therapeutic vaccination with any of our GMTVs had any significant tumour inhibitory effects.

It may be the case that the tumours chosen for the *in vivo* experiments were too malignant to eradicate. For instance, the tumour used in the first experiment was first grown in a SCID/bg mouse prior to the vaccination experiment. This added step likely selected a highly malignant clone that was inherently difficult to kill. Furthermore, the tumour burden imposed on the animals by injection into both flanks may have been too great to overcome. Both of these concerns were addressed in later experiments, but the modifications seemed to make little difference to the overall outcome. Other changes to the experimental design have been proposed that may enhance our ability to detect an effect. These include modifying the vaccination protocol to begin earlier and to use larger vaccine inoculums. Furthermore, more effective immune reconstitution using greater numbers of peripheral blood lymphocytes or perhaps umbilical cord blood lymphocytes (which have proportionally greater numbers of progenitor and naïve cells). These design modifications have not yet been implemented, but future experiments are planned.

Conclusions

This study has demonstrated the feasibility and utility of establishing long-term melanoma cell cultures. Genetically modified tumour vaccines were created using retroviral mediated gene transfer. Melanoma cells transduced to express both GM-CSF

and CD86 (B7-2) demonstrated immune stimulatory properties in an *in vitro* setting.

None of our vaccines demonstrated significant tumour inhibitory properties in an *in vivo* model; however, further modification of this model may eventually demonstrate vaccine efficacy.

Table 2-1. Immunohistochemical analysis of cultured melanoma cells.

CELL	MELAN A	S-100	HMB45
Ed137.Mel	negative	negative	negative
Ed141.Mel	positive	positive	positive
Ed141A1.Mel	positive	positive	positive
Ed153.Mel	negative	positive	positive
Ed156.Mel	negative	negative	negative
Ed157.Mel	negative	negative	negative
Ed162.Mel	negative	positive	positive
Ed165.Mel	negative	negative	negative
Ed169.Mel	negative	negative	negative
Ed176.Mel	negative	negative	negative
Ed178.Mel	negative	negative	negative
SKMel-28	positive	positive	positive

Table 2-2. Expression of common cell surface molecules on cultured melanoma cells.

Cell	HLA Class I	HLA Class II	Fas	Fas-Ligand	CD80 (B7-1)	CD86 (B7-2)
SKMel-28	positive	negative	positive	negative	positive	negative
Ed141.Mel	negative	negative	positive	negative	positive	negative
Ed153.Mel	negative	negative	positive	negative	negative	negative
Ed157.Mel	positive	negative	positive	positive	positive	negative
Ed162.Mel	positive	positive	positive	negative	negative	negative
Ed169.Mel	positive	negative	positive	negative	positive	negative
Ed176.Mel	positive	positive	positive	negative	negative	negative
Ed194.Mel	negative	negative	positive	negative	positive	negative
Ed222.Mel	positive	negative	positive	negative	positive	negative
Ed224.Mel	negative	negative	positive	negative	positive	negative
Ed239.Mel	positive	positive	positive	negative	negative	negative
Ed243.Mel	positive	negative	positive	negative	negative	negative

Table 2-3. Expression of CD86 (B7-2) following retroviral transduction of cultured melanoma cells with pLSN-B70 or pLSN-BG9 vectors.

Cell	CD86 (B7-2) Expression
Ed141.Mel WT (wild type)	negative
Ed141.Mel pLSN B70	positive
Ed141.Mel pLSN BG9	positive
Ed162.Mel WT	negative
Ed162.Mel pLSN B70	positive
Ed162.Mel pLSN BG9	positive
Ed176.Mel WT	negative
Ed176.Mel pLSN B70	positive
Ed176.Mel pLSN BG9	positive
Ed194.Mel WT	negative
Ed194.Mel pLSN B70	positive
Ed194.Mel pLSN BG9	positive
SKMel-28 WT	negative
SKMel-28 pLSN B70	positive
SKMel-28 pLSN BG9	positive

Table 2-4. GM-CSF levels (as determined by ELISA) in cultured melanoma cells transduced with either pLSN-GM1 (a monocistronic vector containing the GM-CSF gene) or pLSN-BG9 (a bicistronic vector containing the genes for CD86 (B7-2) and GM-CSF).

Cell	GM-CSF (pg/10 ⁶ cells/ml)
Ed141.Mel WT (wild type)	8.1 +/- 9.3
Ed141.Mel pLSN GM1	162757 +/- 56252
Ed141.Mel pLSN BG9	1986.7 +/- 290.9
Ed162.Mel WT	0.3
Ed162.Mel pLSN GM1	13488.3
Ed162.Mel pLSN BG9	28.0

Table 2-5. IL-12 levels (as determined by ELISA) in cultured melanoma cells transduced with pLSN-IL12 (a bicistronic vector containing the genes for the p40 and p35 subunits of IL-12).

Cell	IL-12 (pg/10 ⁶ cells/ml)
Ed141.Mel WT (wild type)	3.8 +/- 5.4
Ed141.Mel pLSN IL12	102.7 +/- 93.0
Ed162.Mel WT	0.2 +/- 0.2
Ed162.Mel pLSN IL12	185.5 +/- 64.3

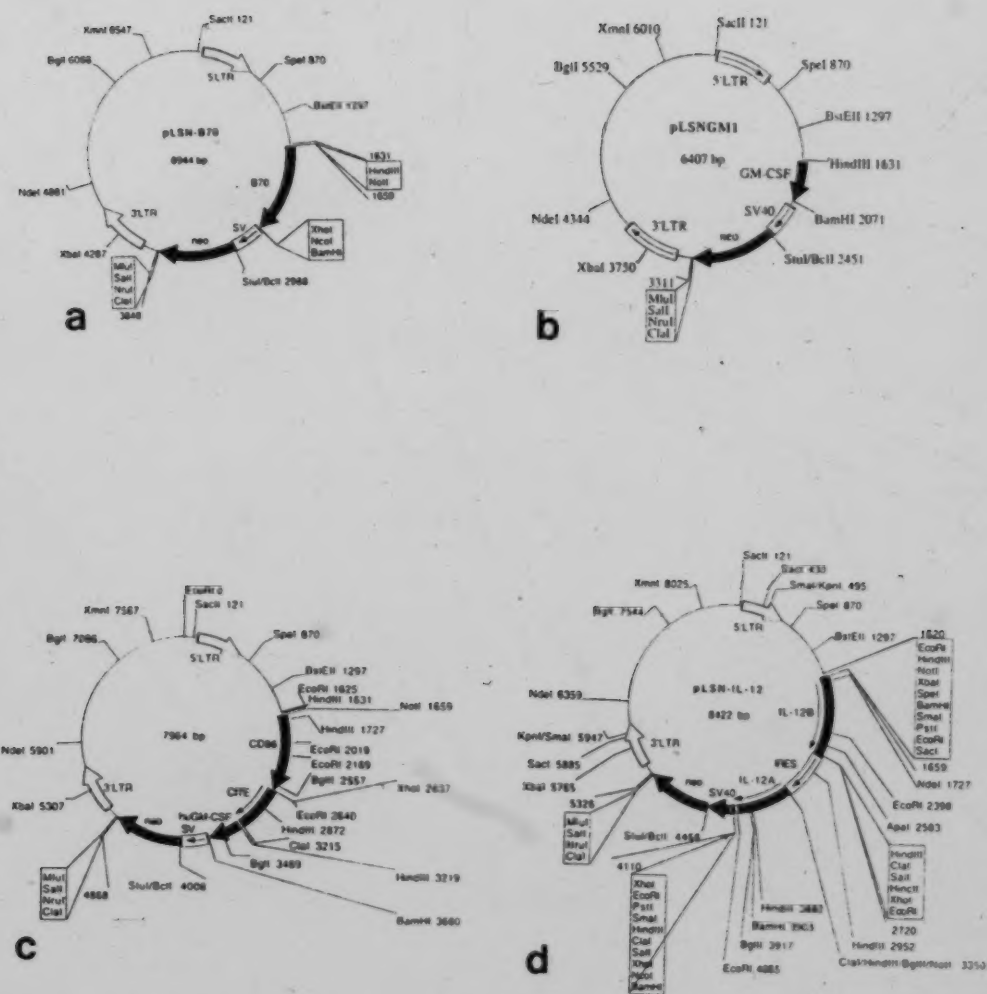


Figure 2-1. Diagrammatic representations of the retroviral vectors: a) pLSN-B70, b) pLSN-GM1, c) pLSN-BG9, and d) pLSN-IL12.

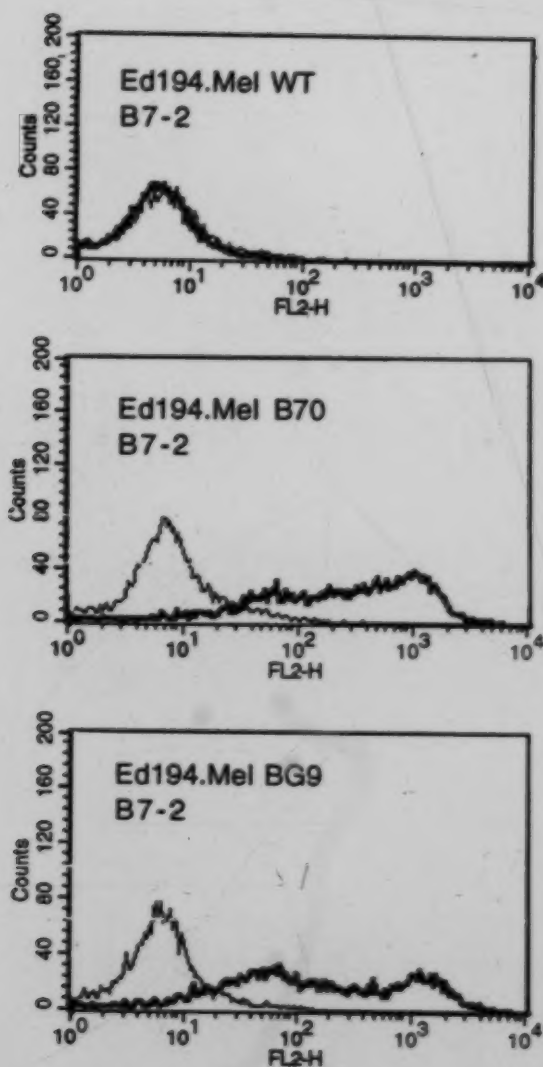


Figure 2-2. Flow cytometry histograms demonstrating successful transduction of CD86 into Ed194.Mel cells using the retroviral vectors pLSN-B70, a monocistronic vector encoding the gene for CD86, or pLSN-BG9, a bicistronic vector encoding the genes for CD86 and GM-CSF.

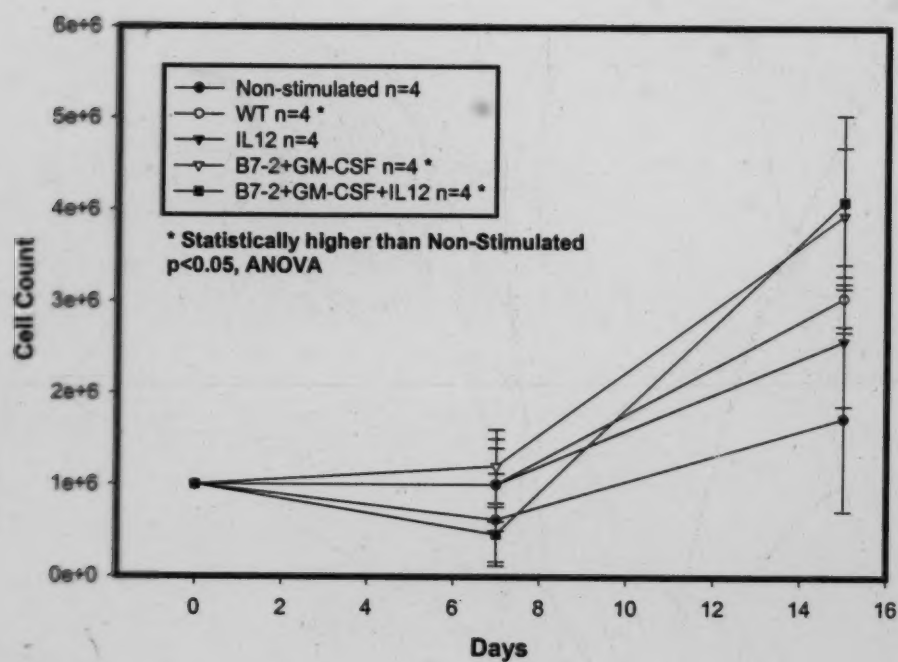


Figure 2-3. The proliferative response of peripheral blood mononuclear cells (PBMC) to stimulation with wild type and transduced forms of Ed141. Mel (Ed141.Mel pLSN-GM1, Ed141.Mel pLSN-IL12, Ed141.Mel pLSN-BG9, and a 1:1 mix of Ed141.Mel pLSN-BG9 and Ed141.Mel pLSN-IL12 ("triple vaccine")).

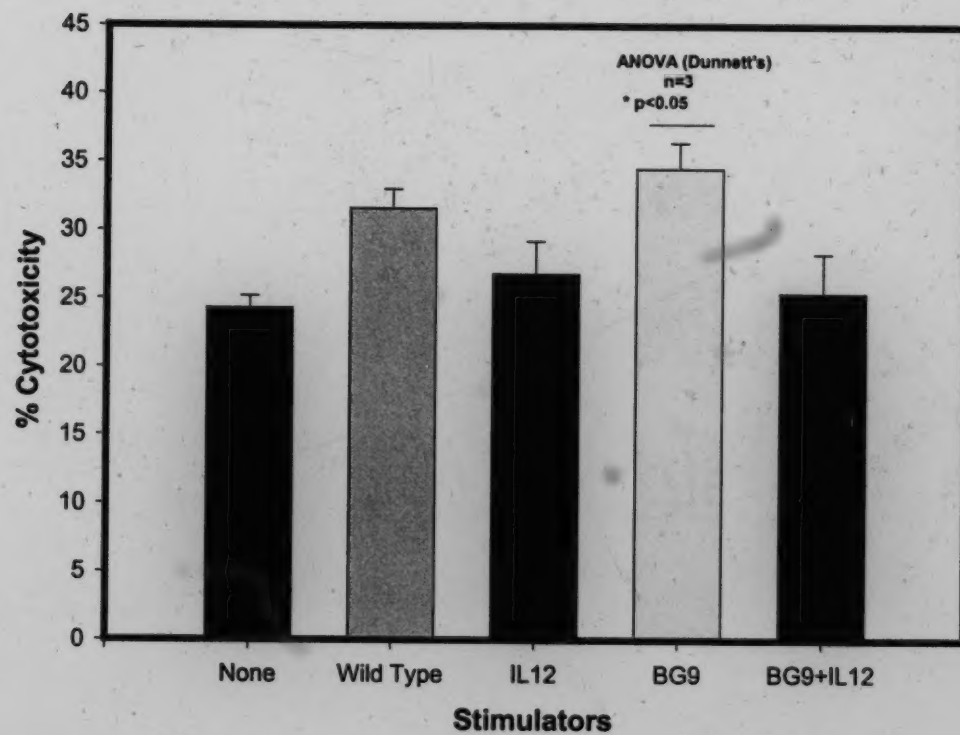


Figure 2-4. Increased cytotoxic potential of peripheral blood mononuclear cells (PBMC) following stimulation with wild type and transduced Ed141.Mel cells.

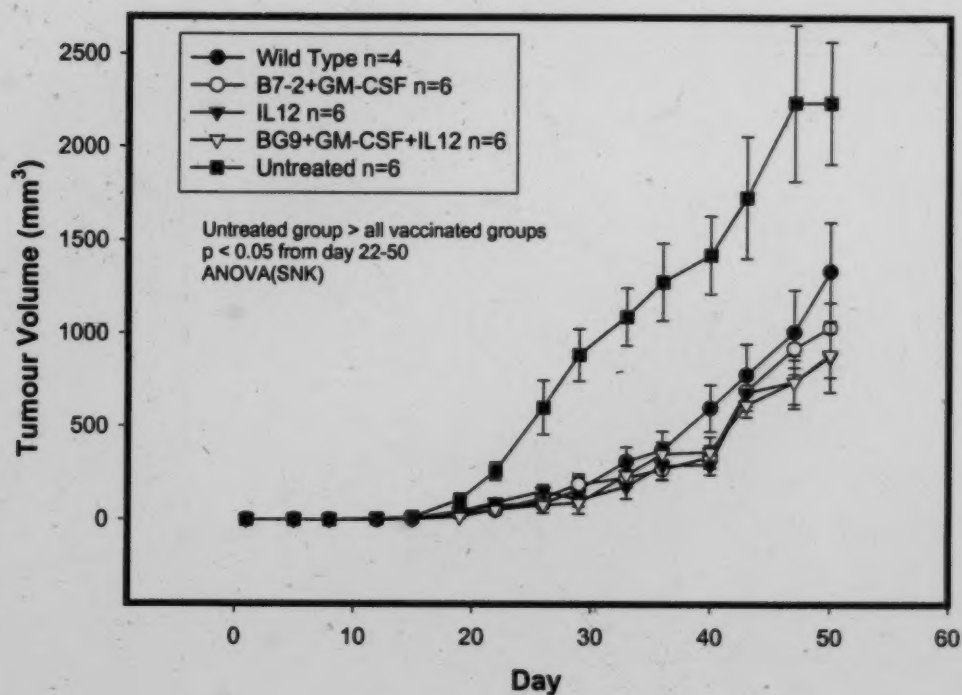


Figure 2-5. Growth of human melanoma (Ed141A1.Mel) in vaccinated, immune-reconstituted SCID/bg mice. Ed141A1.Mel is a highly malignant tumour that had previously been grown in SCID/bg mice, harvested, and then re-established in tissue culture. A single vaccination at day 17 was performed.

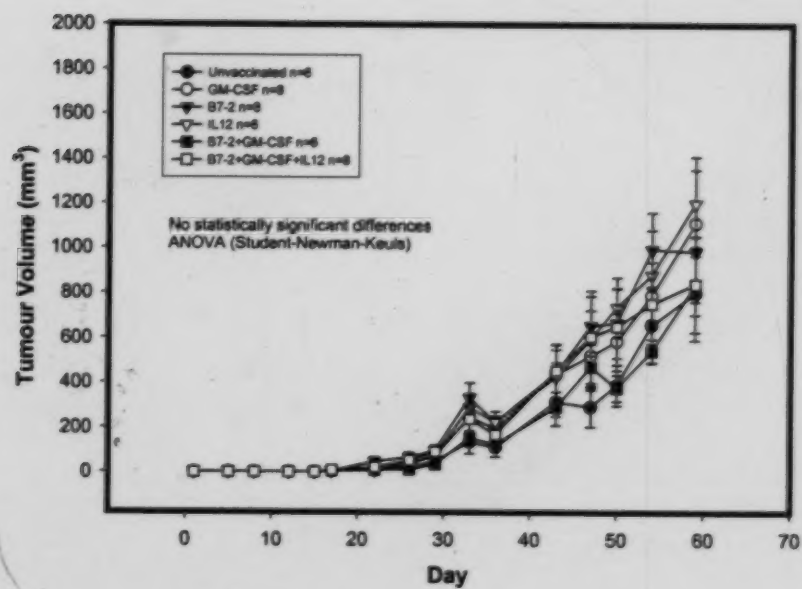


Figure 2-6. Growth of human melanoma (Ed141.Mel) in vaccinated, immune-reconstituted SCID/bg mice. Two vaccinations were administered at days 14 and 21.

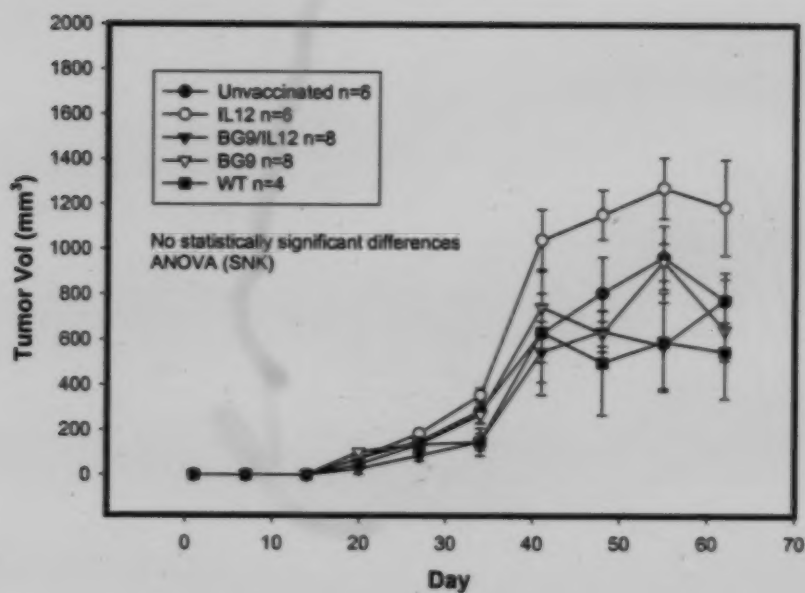


Figure 2-7. Growth of human melanoma (Ed162.Mel) in vaccinated, immune-reconstituted SCID/bg mice. Two vaccinations were administered at days 14 and 21. Ed162.Mel expresses HLA Class I molecules.

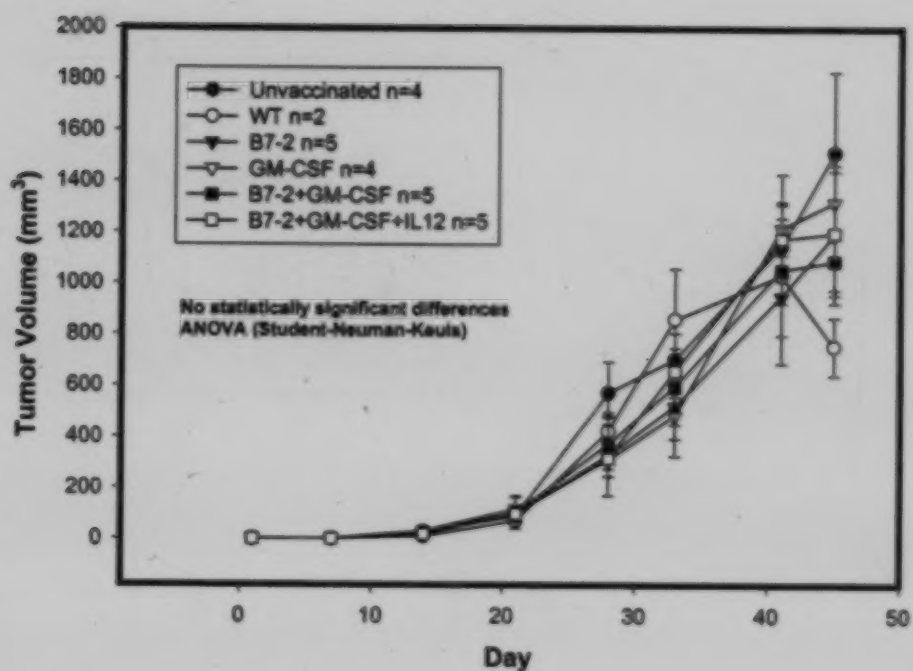


Figure 2-8. Growth of human melanoma (Ed176.Mel) in vaccinated, immune-reconstituted SCID/bg mice. Two vaccinations were administered at days 14 and 21. Ed176.Mel expresses HLA Class I molecules.

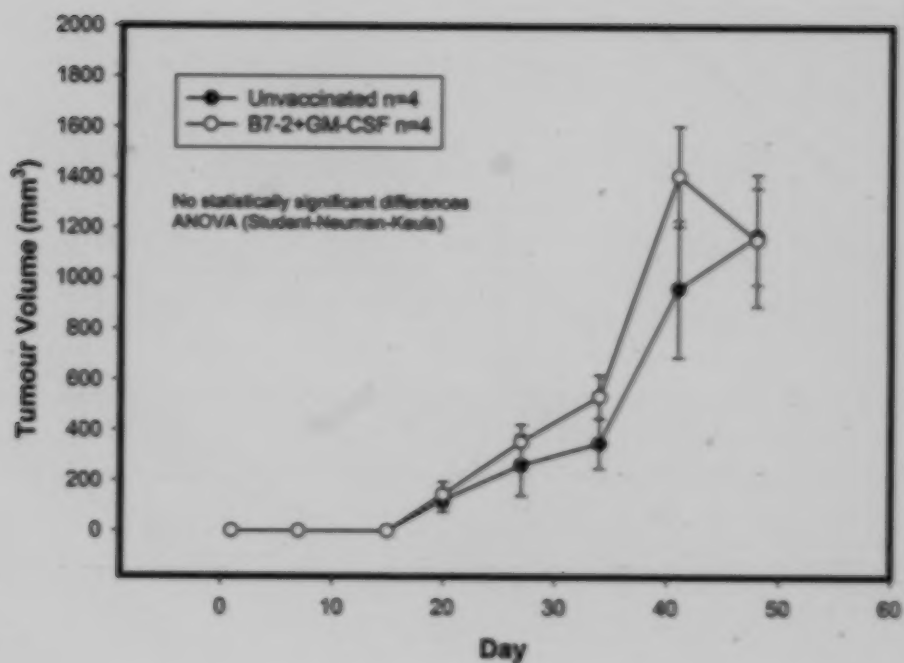


Figure 2-9. Growth of human melanoma (Ed176.Mel) in vaccinated, immune-reconstituted SCID/bg mice. Unlike the other experiments, this was an autologous system in that the peripheral blood lymphocytes used for immune reconstitution were from the patient that Ed176.Mel was harvested from. Two vaccinations were administered at days 14 and 21.

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Chapter 3

Retroviral-Mediated Transduction of Human Melanoma Cells With CD154

Introduction

Malignant melanoma is a malignancy with a propensity for spread beyond the confines of the original tumour. Once metastatic, the prognosis is dismal with a median survival of 6-8 months ([1], [2], [3]). As such, effective adjuvant systemic therapy for this disease would be desirable. Traditional chemotherapy regimens have been disappointing ([4]). Only interferon (IFN)- α 2b has demonstrated the capacity to improve survival in end-stage melanoma ([5]). Numerous other immune-based therapies are being studied, but as yet none have reached widespread clinical applicability.

Our laboratory is interested in creating autologous genetically modified tumour vaccines that serve to upregulate an anti-tumour immune response. We have previously looked at the anti-tumour capabilities of vaccines expressing granulocyte macrophage-colony stimulating factor (GM-CSF) and the co-stimulatory molecule CD86 (B7-2) ([6]). This study explores the role of the CD40:CD154 interaction, which is purported to be a key interaction in cell-mediated immune responses ([7], [8]). Specifically, we have developed a genetically modified tumour vaccine consisting of melanoma cells retrovirally transduced to express CD154 with the objective of an enhanced immune response against melanoma cells.

The CD40:CD154 receptor-ligand pair is related to the tumour necrosis factor receptor (TNFR):tumour necrosis factor (TNF) family of receptor ligand pairings. Both are transmembrane proteins. CD40 has been detected on numerous cell types such as dendritic cells (DC) ([9], [10]), monocytes ([11]), macrophages ([12]), B-cells ([9]), fibroblasts ([12]), vascular endothelial cells([13] [14]), and thymic epithelial cells ([15]). The natural ligand for CD40 is CD154 (sometimes referred to as CD40-Ligand) ([16]).

Although the predominant expression of CD154 is on activated CD4⁺ T-helper cells ([17]), this molecule has also been demonstrated to be present on mast cells, eosinophils, and basophils ([18]).

The interaction most relevant to our study is between CD40 on antigen presenting cells (APCs) (DC, in particular) and CD154 on CD4⁺ T-helper cells, which represents an intermediary step in the process of CD8⁺ cytotoxic T-lymphocyte (CTL) activation ([19], [20], [21], [10]). DCs encounter antigens at the site of injury, infection, or tumour. These antigens are captured, processed, and then presented on DC cell surfaces in conjunction with Class I and Class II HLA molecules. Such cells migrate to lymph nodes where they can present antigens to naive CD4⁺ and CD8⁺ T-cells. The T-cell receptor (TCR) recognizes and interacts with the antigen-HLA complex. On CD4⁺ T-helper cells, signal 1 ligation leads to upregulation of CD154, which can then bind to CD40 on DCs. CD40 binding leads to upregulation of co-stimulatory molecules, such as CD80 (B7-1) and CD86 (B7-2) ([9], [22]) on DCs. These molecules bind to CD28 on both CD4⁺ and CD8⁺ T-cells, thus conferring the second activating signal. The above description suggests that a simultaneous 3 cell interaction occurs between a CD4⁺ T-helper cell, a DC, and a CD8⁺ T-killer cell, each having the same or similar antigenic specificity. Ridge et al ([19]) argue that such an interaction would be rare. They propose an alternative theory ("The Licensing Theory") which states that a DC first interacts with a CD4⁺ T-helper cell and that this interaction "primes" or "conditions" the DC as described above. Thus, the DC becomes "licensed" to activate countless CD8⁺ T-killer cells. Such an interaction is more probable and would also lead to greater amplification and prolongation of the activation signal.

Further effects of CD40 ligation are to promote DC secretion of interleukin-12 (IL12) (which further stimulates T-cells and promotes a T_h1 -type cell-mediated response ([23], [10], [24])), and DC rescue from apoptosis ([25], [26]).

Our vaccine consists of irradiated melanoma cells, which have been genetically modified to express CD154 on their cell surfaces. These cells serve as both as source of antigen and CD154 for APC conditioning, thus bypassing the requirement for $CD4^+$ T-cells. Our hypothesis is that such a vaccine can be used to "prime" APCs, which are subsequently "licensed" to activate a T-killer response against melanoma cells.

Methods

Cloning CD154

A detailed description of the cloning methods used is outlined in Appendices 2 and 3. CD154 has been previously cloned and the sequence published ([27]). 5' and 3' primers were designed based on this sequence. cDNA was generated using the technique of RT-PCR applied against mRNA isolated from stimulated Jurkat cells (kindly provided by Dr. J.F. Elliott, Dept. of Immunology, The University of Alberta). First strand cDNA was generated using the First-Strand cDNA synthesis kit (Pharmacia Biotech; Baie d'Urfe, Quebec). Initially, CD154 cDNA was then isolated and amplified by pfu polymerase-based PCR using the following primers:

5' PRIMER: 5'-CTCTCTGCGGCCCGCCACCATGATCGAAACATACAACCA-3'

and

3' PRIMER: 5'-CTCTCTGCGGCCGCTCAGAGTTTGAGTAAGCCA-3'

These primers were designed to add the restriction enzyme site (RES) for Mlu-1 to the 5'-end and the RES for Not-1 to the 3'-end. This allowed force cloning into the mammalian expression vector pCSD7 (Fig. 3-2). As an initial cloning exercise, pCSD7-CD154 was used to transfect COS cells using standard Calcium Phosphate transfection methods. Gene expression was confirmed via immunofluorescence staining.

Subsequently, CD154 was re-cloned using primers designed to incorporate the RES for Not-1 to the 5' end and the RES for BamH1 to the 3' end. This was necessary to allow force cloning into the retroviral vector pLSN (see next section). This was performed using the following primers:

5' PRIMER: 5' -CTCTCTGCGGCCCGCCACCATGATCGAAACATACAACCA- 3'

and

3'PRIMER: 5'-CTCTCTGGATCCTCAGAGTTTGAGTAAGCCA- 3'.

PCR amplification was performed on a thermal cycler using the following program: 1 cycle of 94°C x 3 minutes, followed by 32 cycles of 94°C x 1 minute, 55°C x 50 seconds, and 72°C x 2 minutes. PCR products were analyzed with gel electrophoresis using low melting point agarose and isolated using GENE CLEAN (Bio101; Carlsbad, CA).

Generation of a retroviral plasmid vector

The retroviral gene therapy vector pLSN-BG9 was kindly provided to our lab by Dr. L.J. Chang (The University of Florida). This vector is based on the modified murine Moloney leukemia virus ([28]). It is replication incompetent, lacking the *gag*, *pol*, and *env* genes and contains a neomycin resistance gene driven by an SV40 promoter and an

insert containing the genes for GM-CSF and CD86 (B7-2) driven by the retroviral LTR promoter (Fig. 3-3). CD154 cDNA was force-cloned into this vector by removing the BG9 insert with Not I and Bam HI (New England BioLabs; Mississauga, Ontario) and then ligating the CD154 cDNA into the open pLSN plasmid. Amplification of pLSN-CD154 was accomplished by bacterial transformation of DH5 α cells and a Maxi-Prep using EndoFree Plasmid Maxi Kit (QIAGEN; Mississauga, Ontario).

Retroviral plasmid DNA was transfected into the packaging cell line PA317 (ATCC; Rockville, Maryland) using Calcium Chloride transfection methods. Following antibiotic selection with the neomycin analogue G418, infectious replication incompetent retrovirus was harvested from the supernatant of transfected PA317 cultures. The retrovirus was then introduced into the packaging cell line PG13 (ATCC) with the aid of polybrene (see below). Following further selection with G418, replication incompetent retrovirus was harvested from the supernatant of PG13 cultures and immediately frozen at -70°C for later use. The retroviral vector was labelled pLSN-CD154.

Melanoma Cell Culture

The melanoma cell line SKMel28 was purchased from ATCC (HTB-72). Six other melanoma cultures were established from tumours excised from local patients as described elsewhere (Chapter 2, Methods). Briefly, tumour samples were minced into <5mm diameter pieces and digested with 2 mg/ml Collagenase II (GibcoBRL; Burlington, Ontario) and 0.1 mg/ml Hyaluroninase (Roche; Laval, Quebec) in Hank's buffered saline solution (HBSS) (GibcoBRL) with gentle stirring at room temperature for 1 to 3 hours. The resulting suspension was filtered through a tissue culture sieve and

centrifuged over Ficoll-Paque density gradient media (Pharmacia Biotech) at 400 x g for 30 minutes. The cells at the interface were harvested, washed twice with HBSS, and plated onto T25 tissue culture flasks (Corning; Acton, Massachusetts). Cells were grown at 37°C, 5% CO₂ in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 I.U./ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Supplemented media is hereafter referred to as complete media. (Media and supplements were purchased from GibcoBRL)

Retroviral transduction of Melanoma Cells

Wild-type tumour cells were grown in T25 flasks. When they reached the logarithmic growth phase (≈70% confluence), they were exposed to thawed virus and polybrene (4µg/ml) in a volume of 1.5 mL of complete RPMI-1640 media. These cells were incubated at 37°C, 5% CO₂ for 3 to 24 hours, afterwhich fresh media was added. The following day, complete RPMI-1640 containing the neomycin analogue G418 ((250-1000 mg/ml) was added, resulting in the demise of untransduced cells. Selection was complete by 5 to 10 days. Complete RPMI-1640 media (less G418) was added and the cells were allowed to grow to adequate numbers for freezing and other testing.

Cell expression of CD154 was confirmed using immunohistochemistry in all cases. The antibodies used were as follows: anti-human CD154 (PharMingen; Mississauga, Ontario), anti-human CD40 (PharMingen), anti-A. niger enzyme (Dako; Mississauga, Ontario) as an isotype-matched negative control antibody, and a biotinylated anti-mouse IgG secondary antibody (Vector; Burlingame, California).

Immunohistochemical staining was carried out using Vectastain ABC (Vector) as per manufacturer's instructions.

Melanoma-Peripheral Blood Mononuclear Cell Co-Culture

Peripheral Blood Mononuclear cells (PBMCs) were isolated from buffy coat samples obtained from the Canadian Red Cross/Canadian Blood Services by Ficoll-Paque (Pharmacia Biotech) density gradient centrifugation. These cells were immediately frozen at -70°C for later use.

5×10^4 melanoma cells were plated onto individual wells of a 24-well tissue culture plate and irradiated with 20,000 rads in order to halt replication. 1×10^6 thawed PBMCs were then added to each well. The co-cultures were incubated in complete RPMI-1640 media supplemented with 20 units/mL of interleukin-2 (IL-2). On day 5, the PBMCs were harvested, counted with a hemocytometer and trypan blue staining, then replated onto new 24-well plates containing new, freshly irradiated melanoma cells. At day 10, the PBMCs were harvested, counted, and analyzed for expression of CD3, CD4, CD8, and CD56 by flow cytometry (FACScan, Becton-Dickinson; Sparks, Maryland). The antibodies used were: anti-human CD3-FITC/CD56-PE (PharMingen), anti-human CD4-FITC (PharMingen), and anti-human CD8-PE (PharMingen).

Melanoma-Dendritic Cell Co-Culture

Dendritic cells (DC) were isolated from human umbilical cord blood using a modification of published protocols ([29] [30]). Umbilical cord blood was collected as per hospital protocol and generously provided to our lab by Dr. Akabutu (Director of the

Cord Blood Bank, The University of Alberta). Following a 1:1 dilution with HBSS, the cord blood sample was centrifuged over a Ficoll-Paque (Pharmacia Biotech) density gradient. The interface layer containing cord blood mononuclear cells (CBMNCs) was harvested and washed twice with HBSS. CD34⁺ cells were isolated from this mixture using Dynabeads M-450 CD34 (Dynal; Lake Success, New York). These anti-CD34 bearing paramagnetic beads were incubated with the CBMNCs and then rosetted using a magnetic particle concentrator (Dynal). The CD34-bearing cells were released into suspension using DETACHaBEAD CD34 (Dynal), washed twice in HBSS, and then plated onto 6-well plates at concentrations between 5×10^4 - 1×10^5 cells/ml of complete RPMI-1640. GM-CSF (Sigma) was added at a concentration of 200 units/ml and TNF- α (PharMingen) at a concentration of 50 units/ml. Fresh media containing GM-CSF and TNF α was added every 3-5 days.

1×10^6 day 12 DCs were added to 5×10^4 irradiated melanoma cells and co-cultured in complete RPMI-1640 media, GM-CSF (200 units/ml), and TNF- α (50 units/ml) at 37°C, 5% CO₂ for 3 days. DCs largely remained in suspension while melanoma cells remain adherent. The stimulated DC were harvested and stained for expression of Class I and Class II HLA, CD80 (B7-1), and CD86 (B7-2) by flow cytometry. The following antibodies were used: anti-human Class I HLA-FITC (Caltag; Hornby, Ontario), anti-human HLA-DR-FITC (Caltag), anti-human CD80-FITC (ID Labs) and anti-human CD86-RPE (ID Labs).

Results

Cloning CD154 and Generation of a Retroviral Plasmid Vector

The PCR product is visualized on a gel (Fig. 3-1) as an 817 bp fragment. This was sequenced and proved to be a 100% match with the published sequence. Figure 3-4 shows the entire sequence including the restriction enzyme sites at the 5'- and 3'- ends.

Retroviral Transduction of Cultured Melanoma Cells

One commercially obtained established melanoma cell line (SKMel28) and six melanoma cultures established from locally resected patient tumours (Ed141.Mel, Ed176.Mel, Ed222.Mel, Ed224.Mel, Ed239.Mel, Ed243.Mel) were successfully transduced with pLSN-CD154. Ed141.Mel and Ed176.Mel are considered established cell lines as they both have survived greater than 20 passages in tissue culture. The other 4 melanoma patient cultures are considered to be early passage tumour explants as they had been in tissue culture for fewer than 10 passages.

Table 3-1 shows the results of immunohistochemical staining for CD40 and CD154 in wild-type and transduced melanoma cultures. The wild-type forms of the 7 transduced cultured melanoma cells demonstrated no constitutive expression of either CD40 or CD154. In contrast, all 7 transduced cell types had positive immunohistochemistry for CD154 and negative immunohistochemistry for CD40 expression. Figures 3-5 to 3-9 show representative staining patterns. The transduced established cell lines SKMel28 pLSN CD154, Ed141.Mel pLSN CD154, and Ed176.Mel pLSN CD154 had an appreciably higher proportion of cells that expressed CD154 than did the transduced early passage melanoma explants (Ed222.Mel pLSN CD154 and Ed224.Mel pLSN CD154). Staining was also more intense with the established cell lines.

The Effect of CD154-bearing Melanoma Cells on PBMCs

Allogeneic PBMCs isolated from buffy coat were co-cultured with irradiated wild-type and transduced forms of SKMel28 and Ed141.Mel. The control group consisted of PBMCs unexposed to melanoma. Fig. 3-10 shows that a PBMC proliferative response results following 10 days of co-culture with melanoma cells. There was anywhere from a 2.4-5 fold increase in responder cells depending on what the stimulator cells were. In contrast, the unstimulated PBMCs remained relatively constant in number. After 10 days, significant differences in the proliferative response were seen between the wild-type and CD154-bearing stimulator cells (Figure 3-11). SKMel28 pLSN CD154 stimulated an increase in cell number from 1×10^6 to 4.9×10^6 cells compared to the increase to 3.1×10^6 cells seen with SKMel28 ($p < 0.005$). Likewise, Ed141.Mel pLSNCD154 stimulated a greater increase in PBMCs than did Ed141.Mel (3.5×10^6 vs 2.4×10^6 , $p < 0.05$).

After stimulation, the PBMCs were harvested and assayed for lymphocyte markers (Table. 3-2). In the unstimulated PBMCs, the lymphocytes present are predominantly CD4⁺ T-cells (43.1% CD4⁺ vs 25.6% CD8⁺). Following exposure to melanoma cells, there is a shift towards CD8⁺ T-cell predominance. Furthermore, exposure to melanoma cells expressing CD154 results in a more profound skewing towards CD8⁺ T-cell predominance compared to stimulation with wild-type melanoma cells. In the case of SKMel28, the proportion of CD8⁺ T-cells increased from 25.6% to 58.4% following stimulation with wild-type SKMel28, and to 65.6% following stimulation with SKMel28 pLSN CD154. Similarly, in the case of Ed141.Mel, wild-type

stimulation resulted in an increase of CD8⁺ T-cells to 43.1%, while stimulation with Ed141.Mel pLSN CD154 increased this proportion to 60.5%.

There also appears to be an increase in CD56⁺ cells (NK cells) following stimulation, but the relationship to CD154 is inconstant (same Table). More CD56⁺ cells resulted following stimulation with Ed141.Mel pLSN CD154 than Ed141.Mel, but the converse was true with SKMel28 pLSN CD154.

The Effect of CD154-bearing Melanoma Cells on DCs

Allogeneic DCs raised from umbilical cord blood were co-cultured with irradiated wild-type and CD154-transduced forms of SKMel28 and Ed141.Mel. The control group consisted of unstimulated DCs.

Table 3-3 shows the effect of stimulation on DC expression of HLA and B7 molecules. Composite flow cytometry histograms are found in Figure 3-12 to 3-14. Compared to an isotype-matched negative control, all groups of dendritic cells, regardless of the mode of stimulation, expressed significantly higher amounts of Class I HLA, Class II HLA, and CD86(B7-2) (ANOVA, $p < 0.05$); however, there were no differences between the groups in terms of percentage of cells expressing any of the three molecules. Additionally, there were no groups that exhibited significant upregulation of CD80 expression above background. Closer examination of the composite flow cytometry histograms reveals that a subpopulation of dendritic cells expressed very high levels of Class II HLA and CD86 following stimulation with CD154 transductants. This phenomenon was not seen in unstimulated DC or those stimulated with WT cells. In order to quantify this subpopulation of cells, an arbitrary level of fluorescence greater

than 200 was set using histogram markers and the percentage of cells beyond this level measured (Table 3-3.b). DC stimulated with CD154-transductants had significantly higher numbers of high expressors of Class I HLA, Class II HLA, and CD86 ($p < 0.05$ for all comparisons) when compared to unstimulated and wild-type stimulated cells, signifying that in these samples, there is generation of a significant number of cells expressing very high levels of HLA and CD86 molecules.

Discussion

The CD40:CD154 interaction is a key step in the generation of cell-mediated and humoral immune responses ([9], [12], [16], [20]). This study documents the creation of a retroviral-based genetically modified melanoma vaccine with immunostimulatory properties which exploits the CD40:CD154 interaction.

Constitutive expression of CD40 and CD154 on melanoma cells has not been well studied. Van den Oord et al ([31]) looked at the expression of these two molecules on paraffin sections of resected melanoma specimens and found that 58% demonstrated *in situ* expression of CD40, and in a subset of CD40⁺ tumours, 44% also expressed CD154. In our sample of 7 melanoma cultures, we found no evidence of CD40 or CD154 expression in wild type cells. It is possible that CD40⁺ or CD154⁺ melanoma cells may have been cloned out or expression downregulated by culture conditions. Conversely, there are non-malignant cells present in paraffin sections and it is difficult to be sure of the true identity of CD40⁺ cells in such a heterogeneous population.

The method chosen to introduce the CD154 gene into melanoma cells was that of retroviral transduction. This allows stable transfer of the CD154 gene (and the neomycin

resistance gene) into the genome of the melanoma cell. Theoretically, the gene should be present for the lifetime of the cell and should be passed onto daughter cells following cell division. Retroviral transduction was not very efficient since only about 5-10% of cells survive antibiotic selection. This is of secondary importance since these cells replicate very quickly and subsequent generations should retain the CD154 gene. Following transduction, antibiotic selection, and expansion, 50-70% of cell populations of the three established cell lines (SKMel28, Ed141.Mel, and Ed176.Mel) expressed CD154 at detectable levels. This proportion of positively staining cells was substantially higher than for the 4 early passage explants (<20%). Higher rates of replication in established cell lines likely contributes to better expression of transduced genes. The corollary to this is that poor expression by transduced early passage explants may limit the clinical application of a vaccine since, ideally, cultured tumour cells are to be transduced shortly after establishing primary cultures, long before the cells can be considered immortal. More work needs to be done to try to improve gene product expression. A stronger promoter, such as a tissue-specific promoter would probably improve expression significantly. For example, Hart ([32]) used the 5'-flanking sequences of the tyrosinase and tyrosinase-related protein 1 genes to drive expression of foreign genes in melanoma cells. Diaz et al ([33]) used a similar strategy incorporating three tyrosinase enhancer elements into a series of modified Moloney murine leukemia virus vectors, which resulted in high titers and tissue specific expression of foreign genes. Another promoter that would likely increase expression would be the CMV promoter, which is also considered to be a strong promoter ([34]).

It has previously been demonstrated that a CTL response could be generated in T-helper depleted populations by stimulating anti-CD40 antibodies ([19], [35], [36]). The melanoma-PBMC co-culture experiment was an attempt to demonstrate that our genetically modified tumour cells could also potentiate a T-cell response. A PBMC sample is a heterogeneous mix of immune cells including T- and B-lymphocytes, natural killer (NK) cells, and various APCs. Thus, all of the elements necessary to generate an immune response are present. Co-culturing with wild-type melanoma cells resulted in T-cell proliferation, particularly of the CD8⁺ subset. This is an expected result since allogeneic wild-type melanoma cells should be recognized as foreign by APCs, CD4⁺ T-cells and CD8⁺ T-cells of appropriate antigenic specificity. Co-culturing with transduced melanoma cells resulted in significantly greater T-cell proliferation, again predominantly in the CD8⁺ T-cell subset. This suggests that the presence of CD154 potentiates the T-cell response. CD154-bearing melanoma cells are efficient stimulators because they serve as both a source of antigen and CD154 for the APC; thus, bypassing the requirement for a CD4⁺ T-helper cell of appropriate antigenic specificity to be in the vicinity of the APC that encountered the antigen. This is, theoretically, a more efficient means of APC priming than a 3-cell interaction.

The melanoma-DC co-culture provides more direct evidence of CD154 activity. CD40 ligation on cultured DC by anti-CD40 antibodies, soluble CD154, or CD154 transfectants has been shown to upregulate IL-12 production ([37] [10] [38]), protect against Fas:Fas ligand mediated apoptosis ([26]), and to upregulate the expression of adhesion molecules such as ICAM-1 and CD44H ([22]) and the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) ([10] [21]). Similarly, we were able to enhance the

functional potential of DC with CD154-bearing melanoma cells. Such cells stimulated a significant subpopulation of DCs to increase their expression of Class I and Class II HLA molecules and CD86 (but not CD80) molecules. This was a remarkable finding given that constitutive expression of these molecules on unstimulated cells is already high. These molecules were not significantly increased with wild-type stimulation.

The finding that constitutive expression of CD86 is high suggests that the DCs are already partially "primed" prior to encountering melanoma cells. DC were raised from CD34⁺ hematopoietic progenitor cells isolated from umbilical cord blood by exposing these cells to GM-CSF and TNF- α . GM-CSF has been shown to have the capacity to increase CD80 and CD86 expression on murine APCs ([39]); thus, even at the low concentrations of GM-CSF present, some APC stimulation may have resulted. Another possibility is that fetal bovine serum (FBS) in the culture media probably acts as another source of foreign antigen that can activate some of the DCs. Luft et al ([40]) have suggested using serum free conditions in which to raise DCs in order to overcome this problem. Even with these limitations, it was still possible to generate a DC response. Theoretically, these "primed" APCs should be more effective in presenting antigens to and activating T-cells.

Our laboratory is concurrently exploring other immunostimulatory molecules for use in vaccine strategies. Specifically, the cytokines GM-CSF and IL-12, as well as the co-stimulatory molecule CD86 have been placed into retroviral vectors and used in combination vaccine strategies. A Health Protection Branch (HPB) sanctioned clinical trial employing a bicistronic vector encoding for both GM-CSF and CD86 targeting both glioblastoma multiforme and malignant melanoma is currently underway (Petruk,

unpublished). A combined immune gene vaccine strategy utilizing CD154, GM-CSF, and B7-2 is currently being studied in the Hu-PBL SCID/bg mouse model.

Conclusions

This paper demonstrates the feasibility of creating a CD154-based genetically modified tumour vaccine by retroviral-mediated transduction of cultured melanoma cells. Such a vaccine possesses potent immunostimulatory properties, with activation of DCs and a subsequent enhanced antitumour response. Gene expression by transduced established melanoma cell lines is high, although expression of CD154 in early passage tumour (ideal for clinical trial application) remains a concern. More work is required to improve improve levels of expression, particularly in early passage tumour cells.

Table 3-1. Expression of CD40 and CD154 on wild type (WT) and CD154-transduced melanoma cells.

Cell	CD40	CD154
SKMel-28	negative	negative
SKMel-28 pLSN-CD154	negative	positive
Ed141.Mel	negative	negative
Ed141.Mel pLSN-CD154	negative	positive
Ed176.Mel	negative	negative
Ed176.Mel pLSN-CD154	negative	positive
Ed222.Mel	negative	negative
Ed222.Mel pLSN-CD154	negative	positive
Ed224.Mel	negative	negative
Ed224.Mel pLSN-CD154	negative	positive
Ed239.Mel	negative	negative
Ed239.Mel pLSN-CD154	negative	positive
Ed243.Mel	negative	negative
Ed243.Mel pLSN-CD154	negative	positive

Table 3-2. Lymphocyte marker expression on peripheral blood mononuclear cells (PBMC) stimulated with wild type or CD154-transduced melanoma cells. The tabulated values represent the proportion of PBMC expressing the individual markers. This was determined by flow cytometry (two colour staining). CD3 and CD56 expression were assayed together and CD4 and CD8 expression were assayed together. The proportion of PBMC expressing CD8 increased following stimulation with transduced cells. There was a corresponding decrease in CD4 expression.

Stimulator	CD3 ⁺	CD56 ⁺	CD4 ⁺	CD8 ⁺
Non-stimulated	62.9%	10.2%	43.1%	25.6%
SKMel-28	46.7	22.4	10.8	58.4
SKMel-28 pLSN-CD154	52.8	17.6	7.7	65.6
Ed141.Mel	50.1	12.4	33.9	43.1
Ed141.Mel pLSN-CD154	57.0	16.5	14.0	60.5

Table 3-3. Expression of HLA and B7 molecules on dendritic cells (DC) following stimulation with wild type melanoma cells or CD154-transductants by flow cytometry. The DC were isolated from umbilical cord blood samples from random healthy donors; thus, the DC are allogeneic to the stimulator melanoma cells.

a) The percentage of cells expressing HLA and B7 molecules following stimulation compared to an isotype-matched control with the background set to 5%. There are no significant differences between groups. (ANOVA, Student-Newman-Keuls)

	Stimulators			p values
	Non-stimulated	WT Melanoma	CD154 Transductants	
Class I HLA	86.3 +/- 11.1	90.0 +/- 9.3	84.4 +/- 15.6	NS
Class II HLA	67.8 +/- 23.6	71.3 +/- 18.8	70.5 +/- 16.2	NS
CD80 (B7-1)	9.8 +/- 6.5	13.2 +/- 7.4	16.1 +/- 6.0	NS
CD86 (B7-2)	64.4 +/- 9.4	72.7 +/- 5.4	68.5 +/- 5.0	NS

b) The percentage of DC fluorescing at levels greater than 200 (high expressors). The percentage of high expressors was higher in the DC stimulated with CD154 transductants for Class I HLA, Class II HLA, and CD86, but not CD80. (ANOVA, Student-Newman-Keuls)

	Stimulators			p values
	Non-stimulated	WT Melanoma	CD154 Transductants	
Class I HLA	1.3 +/- 0.3	3.5 +/- 2.7	7.2 +/- 1.4	p<0.05
Class II HLA	12.9 +/- 7.6	12.0 +/- 4.7	21.8 +/- 3.0	p<0.05
CD80 (B7-1)	0.5 +/- 0.3	0.4 +/- 0.1	0.5 +/- 0.2	NS
CD86 (B7-2)	5.8 +/- 1.2	8.4 +/- 5.0	22.8 +/- 9.9	p<0.05



Figure 3-1. A 1% agarose gel electrophoresis analysis of the PCR product following RT-PCR amplification of CD154 from Jurkat cell mRNA. Lane 1 = 100 bp markers, Lanes 3 and 4 = cloned CD154.

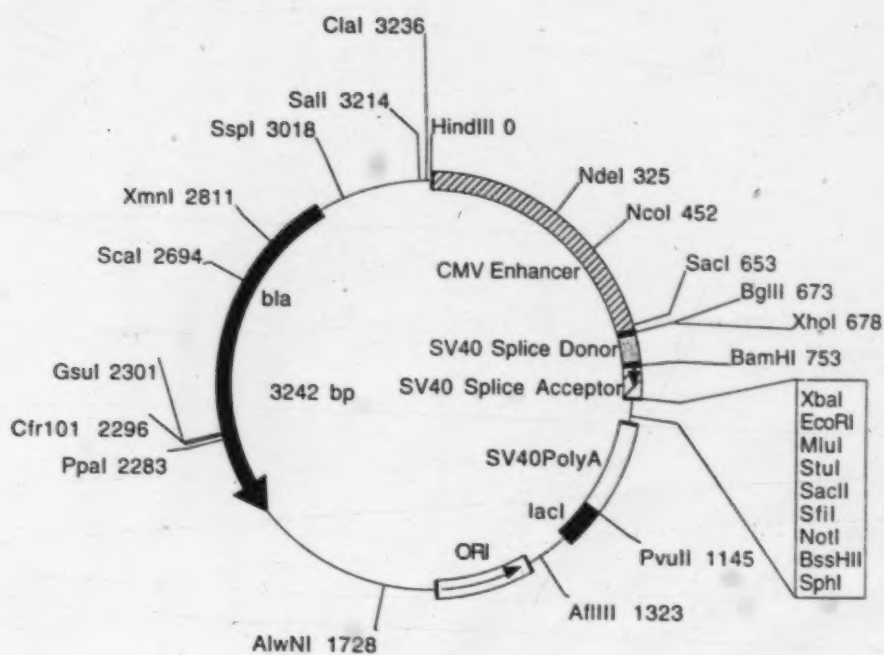


Figure 3-2. Diagrammatic representation of the mammalian expression vector pCSD7 into which the gene for CD154 was first force-cloned. The polylinker site contains the restriction enzyme sites for Mlu-I and Not-I.

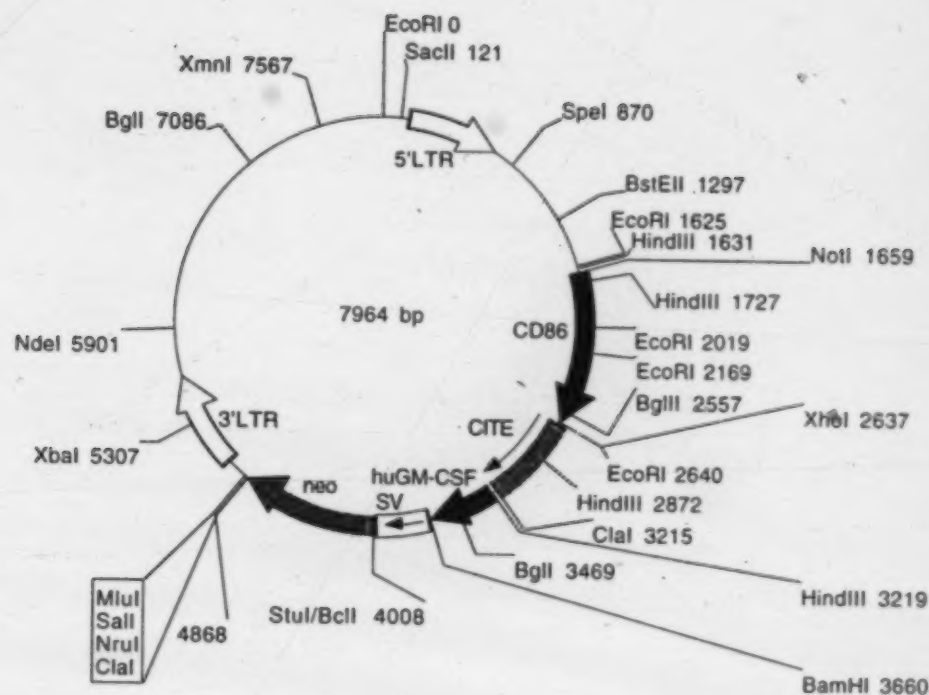


Figure 3-3. A diagrammatic representation of the retroviral vector pLSN-BG9 which contains the genes for B7-2 and GM-CSF. These two genes were spliced out using double digestion with Not-I and BamHI, allowing subsequent insertion of the cloned gene 5'-Not-I - CD154 - BamHI-3'.

5' ctctct**gcggcgcg**ccaccATGATCGAAACATACAACCAA**CTT**
 CTCCCCGATCTGCGGCCACTGGACTGCCCATCAGCATGAAAATTTT
 TATGTATTTACTTACTGTTTTCTTATCACCCAGATGATTGGGTCA
 GCACTTTTTGCTGTGTATCTTCATAGAAGGCTGGACAAGATAGAAG
 ATGAAAGGAATCTTCATGAAGATTTTGTATTTCATGAAAACGATACA
 GAGATGCAACACAGGAGAAAGATCCTTATCCTTACTGAACTGTGAG
 GAGATTAAAAGCCAGTTTGAAGGCTTTGTGAAGGATATAATGTAA
 ACAAGAGGAGACGAAGAAAGAAAACAGCTTTGAAATGCAAAAAGG
 TGATCAGAATCCTCAAATTGCGGCACATGTCATAAGTGAGGCCAGC
 AGTAAACAACATCTGTGTTACAGTGGGCTGAAAAGGATACTACA
 CCATGAGCAACA**ACTTGGTA**ACCCTGGAAAATGGGAAACAGCTGAC
 CGTTAAAAGACAAGGACTCTATTATATCTATGCCCAAGTCACCTTC
 TGTTC**CAATCGGGA**AGCTTCGAGTCAAGCTCCATTTATAGCCAGCC
 TCTGCCTAAAGTCCCCCGGTAGATTTCGAGAGAATCTTACTCAGAGC
 TGCAAATACCCACAGTTCCGCCAAACCTTGCGGGCAACAATCCATT
 CACTTGGGAGGAGTATTTGAATTGCAACCAGGTGCTTCGGTGT**TTG**
 TCAATGTGACTGATCCAAGCCAAGTGAGCCATGGCACTGGCTTCAC
 GTCCTTTGGCTTACTCAA**ACTCTGAggatccagagag**-3'

Figure 3-4. The sequence of cloned CD154. The restriction enzyme sites for NotI and BamHI are sub-cloned into the 5' and 3' sites respectively. They are indicated by boldface type.

a) Ed141.Mel WT



b) Ed141.Mel pLSN CD154

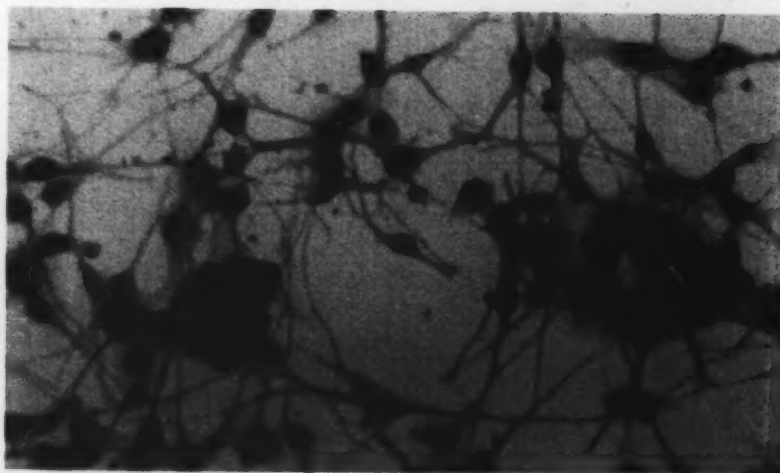


Figure 3-5. CD154 immunohistochemical staining patterns of wild-type (a) and transduced (b) Ed141.Mel cells. A brown staining pattern indicates the presence of CD154. This is seen only in transduced cells (b).

a) SKMel28 WT

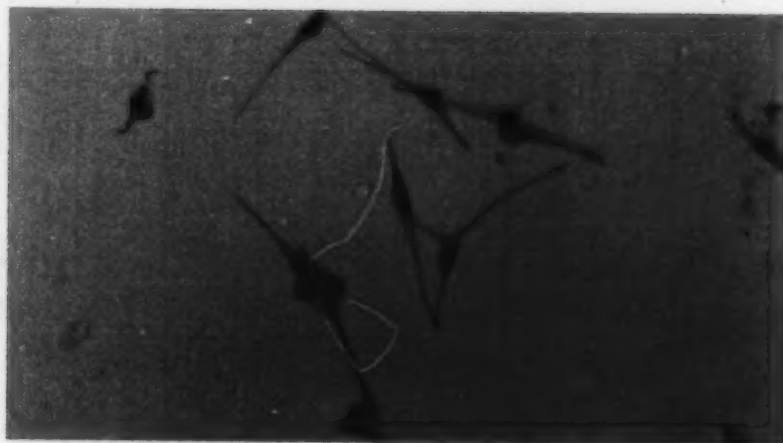


b) SKMel28 pLSN CD154



Figure 3-6. CD154 immunohistochemical staining patterns of wild-type (a) and transduced (b) SKMel-28 cells. A brown staining pattern indicates the presence of CD154. This is seen only in transduced cells (b).

a) Ed176.Mel WT



b) Ed176.Mel pLSN CD154

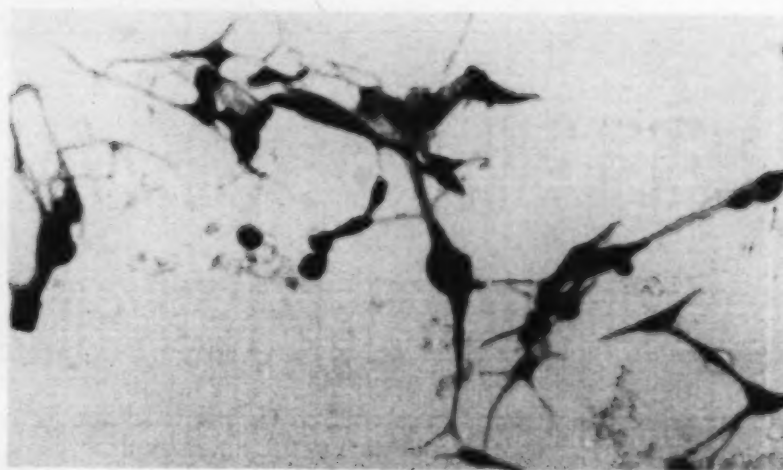
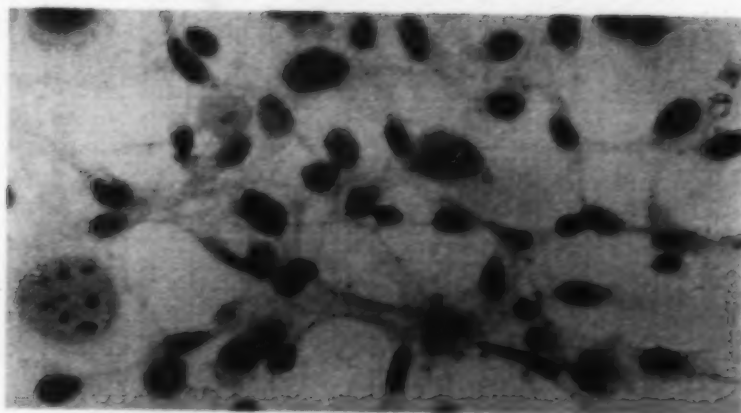


Figure 3-7. CD154 immunohistochemical staining patterns of wild-type (a) and transduced (b) Ed176.Mel cells. A brown staining pattern indicates the presence of CD154. This is seen only in transduced cells (b).

a) Ed222.Mel WT



b) Ed222.Mel pLSN CD154

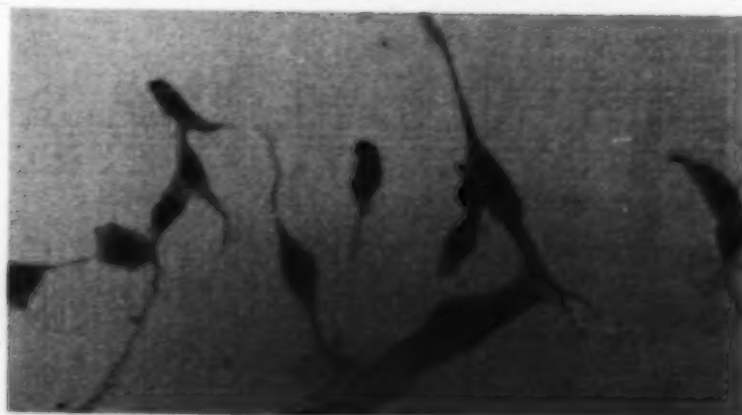
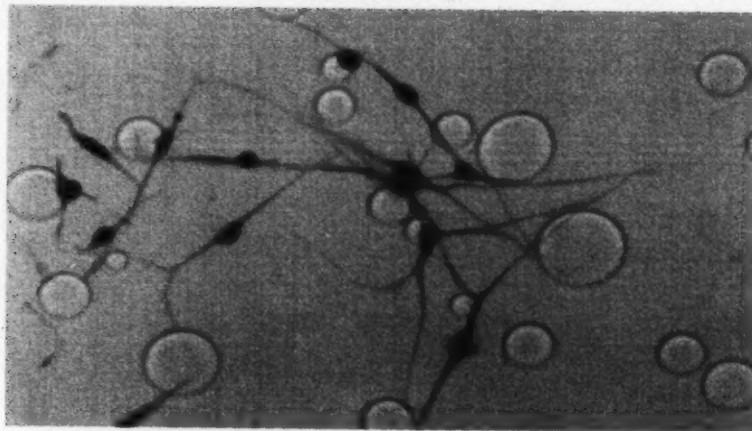


Figure 3-8. CD154 immunohistochemical staining patterns of wild-type (a) and transduced (b) Ed222.Mel cells. A brown staining pattern indicates the presence of CD154. This is seen only in transduced cells (b).

a) Ed224.Mel WT



b) Ed224.Mel pLSN CD154

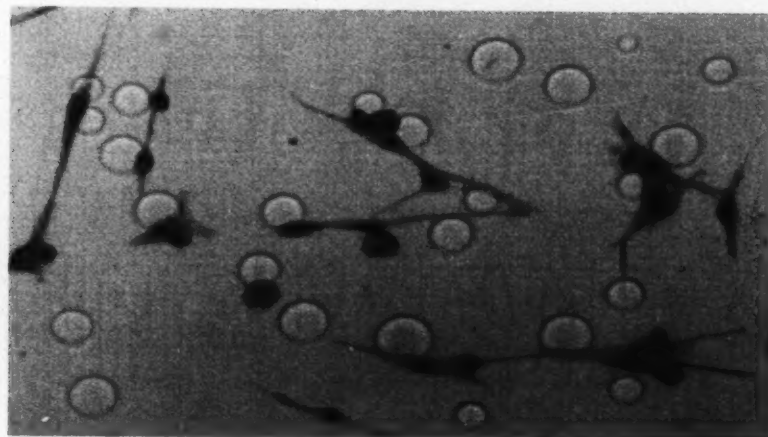


Figure 3-9. CD154 immunohistochemical staining patterns of wild-type (a) and transduced (b) Ed224.Mel cells. A brown staining pattern indicates the presence of CD154. This is seen only in transduced cells (b).

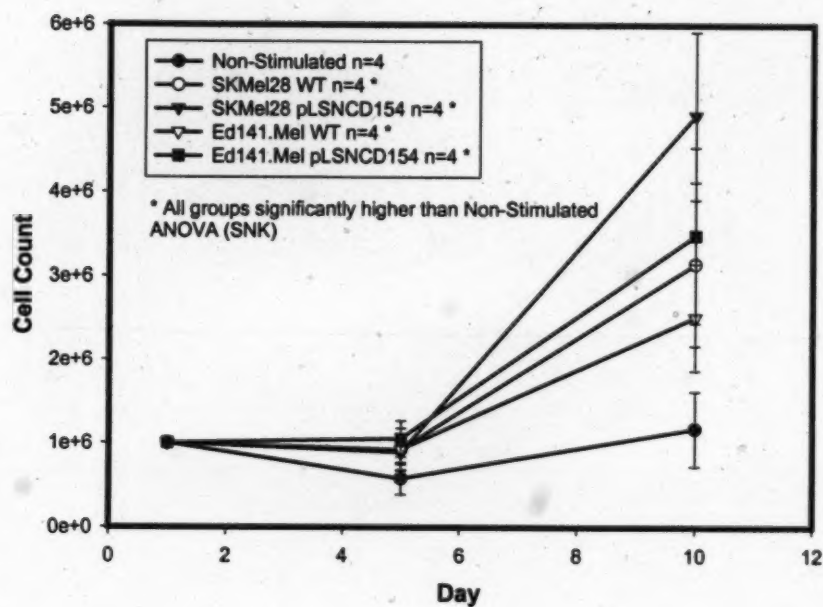


Figure 3-10. Proliferative response of peripheral blood mononuclear cells (PBMC) induced by co-culture with wild type (WT) and transduced melanoma cells. The proliferative response seen in all four groups of stimulated cells was higher than the response in the non-stimulated cells. (ANOVA, Student-Newman-Keuls)

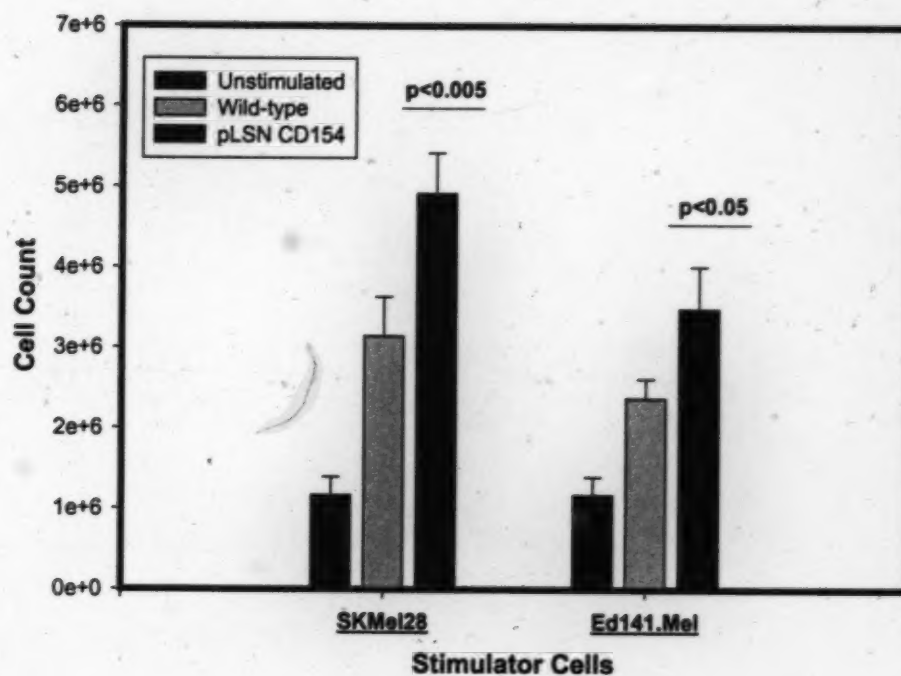
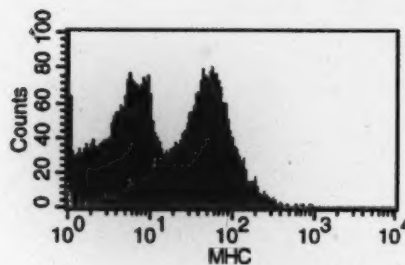
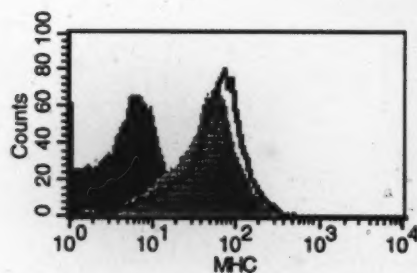


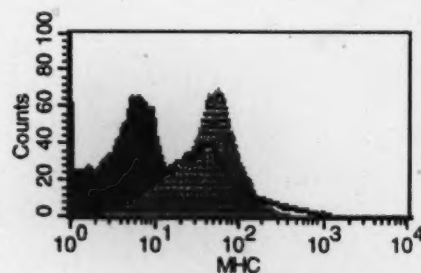
Figure 3-11. Enhancement of the peripheral blood mononuclear cell (PBMC) proliferative response when stimulated with CD154-transductants as compared to stimulation with wild type cells. (ANOVA, $p<0.05$).



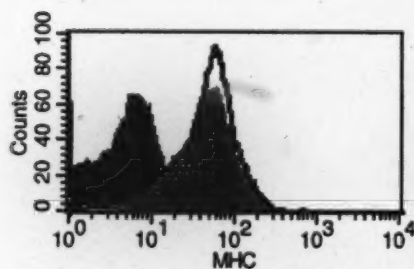
a) Non-stimulated DC control.



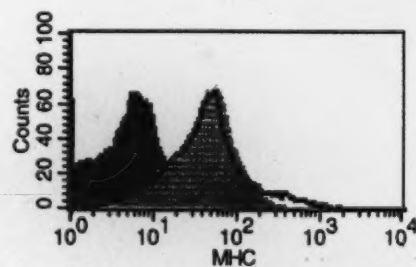
b) DC stimulated with Ed141.Mel-WT.



c) DC stimulated with Ed141.Mel-CD154.



d) DC stimulated with SKMel28-WT.



e) DC stimulated with SKMel28-CD154.

Figure 3-12. Composite flow cytometry histograms of dendritic cell (DC) expression of Class I HLA molecules following co-culture with wild type melanoma cells or CD154-transductants. a) Non-stimulated control, b) DC stimulated with Ed141.Mel-WT, c) DC stimulated with Ed141.Mel-CD154, d) DC stimulated with SKMel28-WT, e) DC stimulated with SKMel28-CD154. (Black=background fluorescence, Grey=non-stimulated DC, Clear=stimulated DC). There is slightly increased expression of Class I HLA molecules following stimulation with the CD154-transductants (c and e).

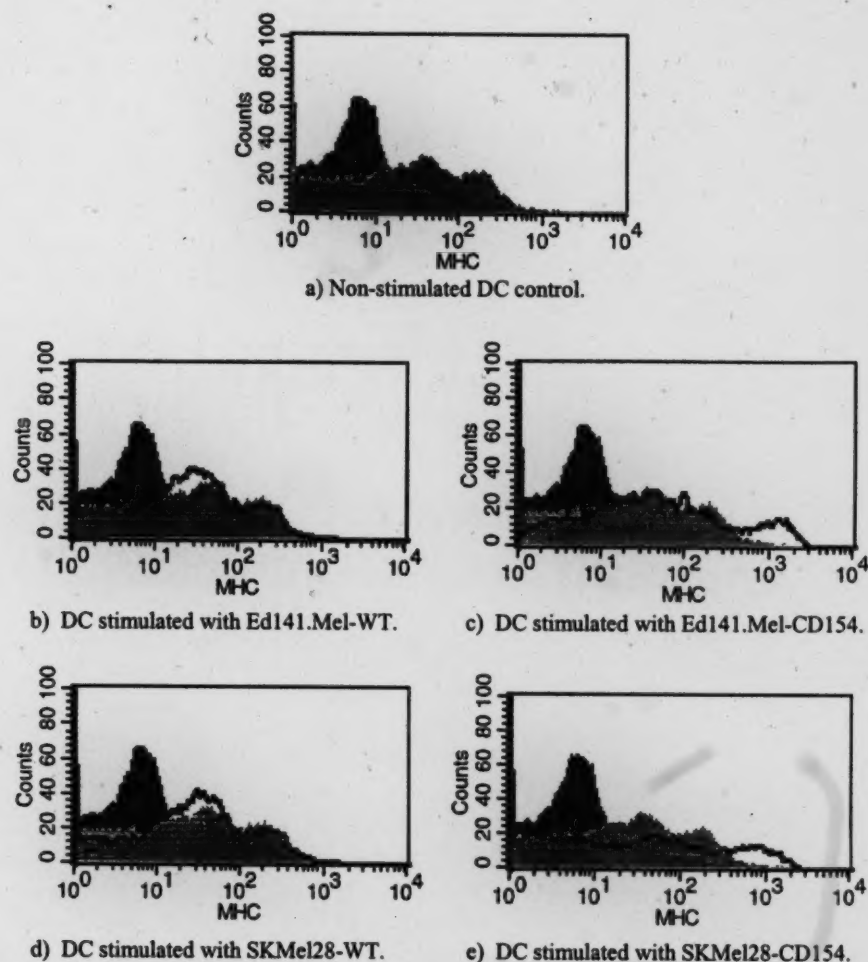


Figure 3-13. Composite flow cytometry histograms of dendritic cell (DC) expression of Class II HLA molecules following co-culture with wild type melanoma cells or CD154-transductants. a) Non-stimulated control, b) DC stimulated with Ed141.Mel-WT, c) DC stimulated with Ed141.Mel-CD154, d) DC stimulated with SKMel28-WT, e) DC stimulated with SKMel28-CD154. (Black=background fluorescence, Grey=non-stimulated DC, Clear=stimulated DC). There is a population of cells with very high expression of Class II HLA molecules following stimulation with the CD154-transductants (c and e).

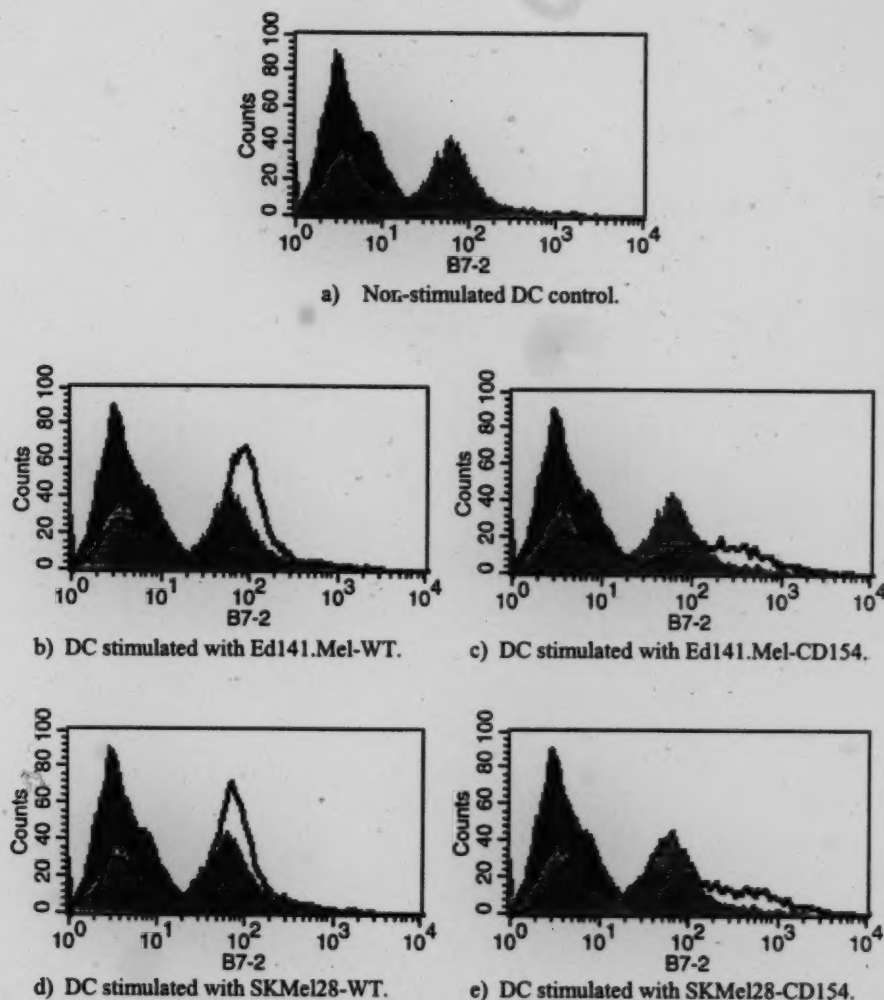


Figure 3-14. Composite flow cytometry histograms of dendritic cell (DC) expression of B7-2 molecules following co-culture with wild type melanoma cells or CD154-transductants. a) Non-stimulated control, b) DC stimulated with Ed141.Mel-WT, c) DC stimulated with Ed141.Mel-CD154, d) DC stimulated with SKMel28-WT, e) DC stimulated with SKMel28-CD154. (Black=background fluorescence, Grey=non-stimulated DC, Clear=stimulated DC). There is a population of cells with very high expression of B7-2 molecules following stimulation with the CD154-transductants (c and e).

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Chapter 4

General Discussion and Conclusions

General Discussion and Conclusions

The rapidly rising incidence of malignant melanoma and the current lack of adequate systemic therapy contribute to make this malignancy a growing problem. Traditional forms of adjuvant therapy, such as radiation and chemotherapy, have not proved to be sufficiently effective ([1], [2]). In the continuing search for more effective forms of treatment, many groups have entertained the notion of immune-based therapies ([3], [4], [5], [6]). Experimental and clinical lines of evidence suggest that melanoma is inherently immunogenic, thus making this malignancy a very attractive target for immune-based strategies ([7]).

Our study concerns itself with one type of immune therapy -- the use of genetically modified tumour cells as therapeutic vaccines. Clinical application of such a strategy would ideally involve surgery to remove the bulk of the tumour, followed by *ex vivo* creation of genetically modified tumour vaccines (GMTVs) by retroviral transduction of cultured tumour cells, and then administration of irradiated GMTVs back to the afflicted patient. It is proposed that GMTVs would upregulate an immune response directed towards antigens specifically associated with that patient's cancer. This project provides important pre-clinical evidence supporting the feasibility of such an approach.

The majority of early work exploring cancer gene therapy has been done using animal analogues of human tumours. In the case of malignant melanoma, the B16 murine melanoma model has been the prototypical study system ([8]). Dramatic tumour inhibition has been demonstrated with adenovirus-based GMTVs, retroviral-based

GMTVs, and others ([9]). However, these studies are specifically limited by the use of murine, rather than human, tumours. With this in mind, our first goal was to establish a human melanoma tissue culture bank. With refinements of previously published protocols ([10]), we were successful in establishing melanoma cultures in the vast majority of cases. This allowed us to study near-homogenous populations of tumour cells, largely free from contaminating cells that might otherwise interfere with interpretation of immunobiologic phenomena. We were able to demonstrate that in a significant number of tested cultures, Class I HLA, Class II HLA, and B7 molecules are not expressed at detectable levels. We reasoned that such cells would be poor antigen presenters and, thus, difficult targets for T-cell responses. However, all of the tested samples did express FAS and none expressed FAS-Ligand. The corollary of these findings is that many melanomas should be susceptible to FAS:FAS-Ligand-mediated killing, while dispelling the notion that surface expression of FAS-Ligand is a common means of immune evasion (at least not in melanoma cultures). Therefore, if these tumours could be made more immunogenic, or if the typically weak native response to them could be strengthened, then these tumours could be successfully eradicated by at least one of the effector arms of the immune system.

While the use of cultured cells was very useful, we remained mindful of the limitations. Some degree of clonal selection occurs in the process of establishing melanoma cultures and cellular phenotypes may change when raised in an *in vitro* setting ([11]). Thus, cultured cells cannot be considered identical to the original tumour cells from which they were raised. Even so, these cells are undoubtedly more accurate representations of *in situ* human tumours than are murine tumours.

Retroviral-mediated transduction was chosen as our method of gene delivery. Our transduction experiments demonstrated that gene transfer was feasible. The main advantage was that gene transfer was stable allowing us to maintain long-term cultures of transduced cells. Cells were sporadically assayed for gene expression weeks to months following transduction confirming continued gene expression. The main disadvantages were the prolonged period of time required to generate large numbers of transduced cells and the lower than expected levels of gene expression seen with our bicistronic constructs. Many of the purported drawbacks such as low efficiency of gene transfer and the requirement for replicating targets, were minor concerns due to the neoplastic nature of our cells. Safety issues continue to remain a concern, but as yet, no clinically significant problems have arisen ([12]).

Our *in vitro* studies looking at the immune-stimulating potentials of our GMTCs were encouraging. In an allogeneic system, we were able to demonstrate significant upregulation of proliferative and cytotoxic capacity of peripheral blood mononuclear cells, which are predominantly T-lymphocytes, with the use of our BG9 vaccine. In other words, provision of B7-2 molecules and GM-CSF upregulated an immune response. Due to the bicistronic nature of our IL-12 construct, IL-12 expression was very low and addition of IL-12 to the *in vitro* systems actually appeared to have an immunosuppressive effect. We postulate that this is a secondary effect from relative overexpression of the p40 subunit of IL-12.

Unfortunately, the encouraging findings from the *in vitro* experiments did not carry over to *in vivo* vaccination experiments in "humanized" SCID/bg mice inoculated with tumour. In 5 separate experiments, no significant tumour inhibitory effects could be

generated with vaccination. Extrapolation of these negative results to a clinical situation would not be valid at this point. The tumours employed in these experiments were likely less immunogenic and, thus, more malignant than the original human tumours that they were derived from. Future modifications to the experimental protocol are planned which may generate a stronger immunostimulatory effect. For example, higher-dose and multiple vaccinations that are instituted earlier are planned for future experiments, as is immune reconstitution of animals with umbilical cord blood rather than peripheral blood. Until these modifications are instituted, the *in vivo* results are best viewed as equivocal.

The most recent development in the project was the addition of CD154 to our armamentarium of immunostimulatory molecules. The gene for CD154 was successfully cloned, inserted into a retroviral vector, and then transferred into cultured human melanoma cells. These CD154-bearing GMTCs showed immune stimulating properties through activation of cultured dendritic cells. Upregulation at this step of the pathway would potentially lead to massive amplification of downstream activation of cytotoxic lymphocytes. Use of this molecule in combination with our other molecules is an exciting proposition. In particular, a combination vaccine expressing both CD154 and GM-CSF would be a highly potent stimulator of antigen presenting cells.

Future Directions

The results of this project are very encouraging and are consistent with published literature on this subject. Though the benefits of using retroviral technology are undeniable, several problems were identified during the course of this project. Foremost was the fact that the processes of transduction and then selection are time consuming. In

the setting of an experimental laboratory, this was largely inconsequential. However, in a clinical setting this becomes very important as patients with advanced disease, for whom the vaccines would be intended, have a very limited life expectancy. Compounding this problem is that safety testing is required due to the theoretical risks of using retroviruses. Many patients would die while awaiting the creation of their vaccines.

Due to this major limitation, alternative methods of gene delivery are being explored. Foremost on the horizon is biolistic gene transfer. Work has been done by our lab and others using a "gene gun" to fire gold particles coated with DNA into cells, thereby transferring genes into cells. This type of gene transfer is epigenetic and is not stable and, eventually, gene expression would be lost. However, for the purposes of vaccination, short-term expression may be all that is required to generate immune upregulation. Bullets coated with GM-CSF, IL-12, B7-2, and CD40 have been transferred into cultured melanoma and glioblastoma cells (Gainer et al, unpublished). This technique is considerably quicker and preliminary data suggests that gene expression is as good or better than that seen following retroviral transduction.

Melanoma was chosen due to its inherent immunogenicity. Neoplasms such as adenocarcinoma, squamous cell carcinoma and others may eventually prove to be appropriate targets as well. Since cell culture and gene transfer techniques are now well established in our lab, few problems would be anticipated if we were to decide to work with other types of tumours.

Conclusions

Our study has demonstrated the feasibility and utility of using cultured melanoma cells. Retroviral-mediated gene transfer of cDNAs encoding GM-CSF, IL-12, B7-2, and CD154 into melanoma was performed. Resulting cells had immunostimulatory properties *in vitro*. Unfortunately, tumour inhibition could not be demonstrated in our *in vivo* Hu-PBL-SCID/bg model. The results of this imperfect model are viewed as equivocal. More work is required to develop a better model in order to more definitively test our genetically modified tumour vaccines in an *in vivo* setting.

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Appendix 1

The American Joint Committee on Cancer Staging System for Cutaneous Melanoma

Primary Tumour (pT)

pTx	Primary tumour cannot be assessed
PT ₀	No evidence of primary tumour
pT _{is}	Melanoma <i>in situ</i> (atypical melanocytic hyperplasia, severe melanocytic dysplasia), not an invasive lesion (Clark's level I)
PT ₁	Tumour thickness 0.75mm or less and invades the papillary dermis (Clark's level II)
PT ₂	Tumour thickness between 0.75-1.5mm and/or invades the reticular dermis (Clark's level III)
pT ₃	Tumour thickness between 1.5-4mm and/or invades the reticular dermis (Clark's level IV)
pT _{3a}	• Tumour between 1.5mm and 3mm in thickness
pT _{3b}	• Tumour between 3mm and 4mm in thickness
pT ₄	Tumour more than 4mm in thickness and/or invades the subcutaneous tissue (Clark's level V) and/or satellite(s) within 2cm of the primary tumour.
pT _{4a}	• Tumour more than 4mm in thickness and /or invades the subcutaneous tissue
pT _{4b}	• Satellite(s) within 2cm of the tumour

Lymph Nodes (N)

N _x	Regional lymph nodes cannot be assessed
N ₀	No regional lymph node metastases
N ₁	Metastases 3cm or less in greatest dimension in any regional lymph node(s)
N ₂	Metastases more than 3cm in greatest dimension in any regional lymph node and/or in-transit metastases
N _{2a}	• Metastases >3cm in greatest dimension in any regional lymph node(s)
N _{2b}	• In-transit metastases
N _{2c}	• Both (N _{2a} and N _{2b})

Distant Metastases (M)

Mx	Presence of distant metastases cannot be assessed
M ₀	No distant metastases
M ₁	Distant metastases
M _{1a}	• Metastases in skin or sub-cutaneous tissue or lymph nodes beyond the regional lymph nodes
M _{1b}	• Visceral metastases

Appendix 1

The American Joint Committee on Cancer Staging System for Cutaneous Melanoma

Stage Grouping

I	pT ₁ , N ₀ , M ₀ pT ₂ , N ₀ , M ₀	Primary melanoma 0.75-1.5mm thick and/or Clark's level II-III; no nodal or systemic metastases
II	pT ₃ , N ₀ , M ₀ pT ₄ , N ₀ , M ₀	Primary melanoma 1.51-4mm thick and/or Clark's level IV-V and/or satellites within 2cm of the primary tumour
III	Any pT, N ₁ , M ₀ Any pT, N ₂ , M ₀	Regional lymph node metastases and/or in-transit metastases; no systemic metastases
IV	Any pT, Any N, M ₁	Systemic metastases

Reference:

Gershenwald J.E., Buzaid A.C., Ross M.I., *Classification and Staging of Melanoma*. Hematology/Oncology Clinics of North America. 12(4):p. 737-765.

Appendix 2: Cloning CD154

Cloning CD154 and generation of the retroviral plasmid vector pLSN-CD154 involved three basic steps. First, the cDNA for CD154 was generated from Jurkat cell mRNA using reverse transcription polymerase chain reaction (RT-PCR). During this step, restriction enzyme sites (RES) were inserted at both the 5' and 3' ends through the use of specifically designed vectors. Step 2 involved splicing of CD154 into a vector by double digestion of the insert and the vector with appropriate RES and then using DNA ligase to ligate the insert into the vector. The final step involved the amplification of pLSN-CD154 by bacterial transformation and overgrowth. The plasmid was then harvested by lysing bacteria and precipitating the plasmid DNA, which was then purified using Qiagen Maxi-Prep columns.

Cloning CD154

CD154 has been previously cloned and the sequence published ([27]). 5' and 3' primers were designed based on this sequence. cDNA was generated using the technique of RT-PCR applied against mRNA isolated from stimulated Jurkat cells (kindly provided by Dr. J.F. Elliott, Dept. of Immunology, The University of Alberta). First strand cDNA was generated using the First-Strand cDNA synthesis kit (Pharmacia Biotech; Baie d'Urfe, Quebec).

The first-strand reaction was performed as per manufacturers instructions. Briefly, 200 nanograms of Jurkat cell mRNA in 8 microlitres of DEPC-treated water was added to 5 microlitres of the first-strand reaction mix (contains Modified Moloney Leukemia virus reverse transcriptase, RNAGuard, RNase-/DNase-free BSA, dATP,

dCTP, dGTP, and dTTP), 1 microlitre of DTT, and 1 microlitre of random hexamer primer. The mixture was then incubated at 37°C for 60 minutes to allow cDNA synthesis. The mixture was then heated to 90°C to allow the strands to separate.

PCR amplification of CD154 was then performed using primers designed to incorporate the RES for Not-1 to the 5' end and the RES for BamH1 to the 3' end. This was necessary to allow for cloning into the retroviral vector pLSN (see next section).

The following primers were used:

5' PRIMER: 5' -CTCTCTGCGGCCGCCACCATGATCGAAACATACAACCA- 3'

and

3' PRIMER: 5' -CTCTCTGGATCCTCAGAGTTTGAGTAAGCCA- 3'.

One microlitre of each primer was added to 15 microlitres of the first strand reaction mix generated from the previous step and 32.7 microlitres of DEPC-treated water. The reaction was catalyzed with 0.3 microlitres of Taq polymerase (New England Biolabs, Mississauga, Ontario) or pfu polymerase (Dr. J.F. Elliott). PCR amplification was performed on a thermal cycler using the following program: 1 cycle of 94°C x 3 minutes, followed by 32 cycles of 94°C x 1 minute, 55°C x 50 seconds, and 72°C x 2 minutes. PCR products were analyzed with gel electrophoresis using low melting point agarose and isolated using GENE CLEAN (Bio101; Carlsbad, CA).

Generation of a retroviral plasmid vector

The retroviral gene therapy vector pLSN-BG9 was kindly provided to our lab by Dr. L.J. Chang (The University of Florida). This vector is based on the modified murine Moloney leukemia virus ([28]). It is replication incompetent, lacking the *gag*, *pol*, and

env genes and contains a neomycin resistance gene driven by an SV40 promoter and an insert containing the genes for GM-CSF and CD86 (B7-2) driven by the retroviral LTR promoter. CD154 cDNA was force-cloned into this vector by splicing the BG9 insert out of pLSN-BG9 with Not I and Bam HI (New England BioLabs) and then ligating the CD154 cDNA into the open pLSN plasmid with T4 DNA ligase (New England Biolabs).

Amplification of pLSN-CD154 was accomplished by bacterial transformation of DH5 α cells. Briefly, 12 microlitres of freshly ligated pLSN-CD154 was added to 50 microlitres of freshly thawed competent DH5 α cells. The mixture was left on ice for 10-20 minutes, then heat-shocked for 90 seconds at 37°C. After placing back on ice for 5 minutes, 1 millilitre of 2XYT media was added and the mixture placed on a rollerwheel for 35 minutes. High and low concentrations were then plated out on carbenicillin-agar plates. Theoretically, only bacteria that contain pLSN-CD154 (which also encodes for an ampicillin resistance gene) should grow. The plates are incubated overnight at 37°C. Colonies were individually picked off of the plates and grown overnight in 5 millilitres of 2XYT media.

Plasmid DNA was purified by using an alkaline lysis mini-prep protocol. Briefly, 4.5 mL of the bacterial culture was spun down resulting in a bacterial pellet. This was resuspended in 150 microlitres of solution I (glucose buffer). The bacteria were lysed with 300 microlitres of solution II (1% SDS-0.2N NaOH), then 200 microlitres of solution III (3M potassium acetate) were added to precipitate contaminants. After centrifugation at 12000 rpm for 5 minutes in a microfuge, the plasmid DNA-containing supernatant was harvested and the plasmid DNA precipitated with an equal volume of isopropanol at -20°C. The precipitated DNA was pelleted at 12000 rpm for 15 minutes,

then washed with 75% EtOH. Following another 12000 rpm spin for 5 minutes, the pellet was dissolved in 50 microlitres of TE buffer containing RNase A. The plasmid DNA was extracted with an equal volume of phenol/chloroform, then precipitated with 0.1 volumes of sodium acetate and 2.2 volumes of EtOH and stored at -20°C overnight. The following day, the DNA was pelleted by centrifugation, resuspended in TE buffer and digested with Not I and BamHI. The digested samples were run on agarose gels confirming the presence of an insert and an open plasmid.

Maxi-preps were carried out on samples with positive mini-preps using EndoFree Plasmid Maxi Kit (QIAGEN; Mississauga, Ontario) as per manufacturers instructions.

Retroviral plasmid DNA was transfected into the packaging cell line PA317 (ATCC; Rockville, Maryland) using Calcium Chloride transfection methods. Briefly, 10 micrograms of plasmid DNA was added to 225 microlitres of double distilled water and 25 microlitres of 2.5M Calcium Chloride. 250 microlitres of BES was slowly added resulting in a fine precipitate. The resulting mixture was then slowly added to PA317 cells growing at 70% confluence in DMEM. The cells were incubated at 37°C , 5% CO_2 for 2 days. Infectious replication incompetent retrovirus was harvested from the supernatant of transfected PA317 cultures in 0.5 mL aliquots and frozen at -70°C or used immediately with the packaging cell line PG13. The retrovirus was introduced into the packaging cell line PG13 (ATCC) with the aid of polybrene. Following selection with G418, replication incompetent retrovirus was harvested from the supernatant of PA317 cultures and immediately frozen at -70°C for later use. The retroviral vector was labelled pLSN-CD154.

Appendix 3: Generation of pCSD7-CD154

Prior to cloning pLSN-CD154, CD154 cDNA was isolated and amplified by pfu-polymerase-based PCR using the following primers:

5' PRIMER: 5'-CTCTCTGCGGCCGCCACCATGATCGAAACATACAACCA- 3'

and

3' PRIMER: 5'-CTCTCTGCGGCCGCTCAGAGTTTGAGTAAGCCA- 3'

These primers were designed to add the restriction enzyme site (RES) for Mlu-1 to the 5'-end and the RES for Not-1 to the 3'-end. This allowed for cloning into the mammalian expression vector pCSD7 (Fig. 3-1) which contains the Mlu-1 and Not-1 RES within its polylinker region. Mini- and maxi-preps were performed as outlined in Appendix 2.

As an initial exercise, pCSD7-CD154 was used to transfect CD154 into green monkey kidney cells using standard Calcium Chloride transfection methods (see Appendix 2) or biolistic gene transfer methods (Dr. A. Gainer). Gene expression was confirmed via immunofluorescence staining with a fluorescently labeled anti-CD154 antibody (Dr. J. Elliott). This gene transfer method was transient and of low efficiency, but it allowed us to quickly confirm that the cloned CD154 gene was transferable into and expressed by mammalian cells. With this at hand, we proceeded to clone CD154 into a retroviral vector (see Appendix 2).